FISH AS NATURAL HOST AND *IN VIVO* MODEL FOR INFECTIONS WITH INTRACELLULAR BACTERIAL PATHOGENS

DISSERTATION

ZUR

ERLANGUNG DER NATURWISSENSCHAFTLICHEN DOKTORWÜRDE

(DR. SC. NAT.)

VORGELEGT DER

MATHEMATISCH-NATURWISSENSCHAFTLICHEN FAKULTÄT

DER

UNIVERSITÄT ZÜRICH

VON

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ZÜRICH, 2015

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II PREFACE

“The proper function of man is to live, not to exist. I shall not waste my days in trying to prolong them. I shall use my time.”

- Jack London -

III SUMMARY

My thesis has focused on naturally occurring bacterial diseases in fish, in particular epitheliocystis and the possibility to use fish, in particular zebrafish, as a model organism to study bacterial diseases *in vivo*. Our studies showed the high divergence of bacterial agents causing epitheliocystis in marine wild and farmed fish species of the Mediterranean. With *Ca*. Syngnamydia venezia we have discovered a new member of the bacterial phylum Chlamydiae, potentially causing epitheliocystis in pipefish and seahorses. Furthermore we have revealed a new genus of beta-proteobacteria, *Ca*. Ichthyocystis spp., as potential agents of the disease in farmed seabream around Greece. One common feature of these bacteria is their intracellular life style. This fact raised the question, whether fish could serve as a general model to study infectious disease caused by this group of pathogens.

To develop such a model system, we have chosen the most widespread and popular fish model in science, the zebrafish. By using different experimental approaches like bath-immersion and microinjection technique to infect zebrafish larvae with *Waddlia chondrophila,* we have established the first zebrafish infection model for an obligate intracellular pathogen and a member of the Chlamydiae. Furthermore, we have developed a second zebrafish larval model for infections with the facultative intracellular pathogen *Cronobacter turicensis*, member of the proteobacteria and an emerging opportunistic pathogen of humans. The application of the novel *in vivo* models gave us the opportunity to investigate the pathogenicity of these bacteria in zebrafish larvae and to compare characteristics of an infection between an obligate and a facultative intracellular pathogen. We discovered virulence traits of both bacteria and tested the efficacy of antimicrobial drugs to treat an infection in our models. Moreover, through the utilisation of transgenic zebrafish lines, we had the ability to observe the innate immune response to infections by either pathogen. By this means we have implemented two new infection models and validated the zebrafish model as a promising and beneficial tool to study bacterial infectious diseases *in vivo*.

IV ZUSAMMENFASSUNG

Meine Arbeit hat sich mit der Untersuchung von bakteriellen Infektionskrankheiten bei Fischen, vor allem Epitheliocystis und der Möglichkeit Fische, wie z.B. den Zebrafisch, als Modellorganismen zur Untersuchung bakterieller Infektionen *in vivo* nutzen zu können beschäftigt. Unsere Untersuchungen von marinen wild lebenden und in Aquakultur gezüchteten Mittelmeerfischen haben eine grosse Vielfalt an bakteriellen Epitheliocystiserregern ergeben. Mit *Ca*. Syngnamydia venezia haben wir einen neuen potentiellen Erreger der Krankheit aus dem Stamm der Chlamydiae bei Seenadeln und Seepferdchen entdeckt. Des Weiteren haben wir mit *Ca*. Ichthyocystis spp. einen neuen Genus von beta-Proteobakterien als mögliche Erreger in Seebrassen rund um Griechenland identifiziert. Eine gemeinsame Eigenschaft dieser Bakterien ist ihre intrazelluläre Lebensweise, was die Frage aufwarf, ob man nicht Fische als ein Modell zur allgemeinen Untersuchung von Infektionen durch diese Pathogene nutzen könnte.

Für die Entwicklung eines solchen Modellsystems haben wir den wohl bekanntesten und in der Wissenschaft am weitesten verbreiteten Modell-Fisch verwendet, den Zebrafisch. Durch die Nutzung verschiedener Methoden wie der Bad-Immersion oder Mikroinjektion zur Infektion von Zebrafisch Larven mit *Waddlia chondrophila*, haben wir das erste Zebrafisch Modell für Infektionen mit einem obligat intrazellulären Pathogen und einem Mitglied der Chlamydiae entwickelt. Darüber hinaus haben wir noch ein zweites Zebrafisch-Modell für Infektionen mit *Cronobacter turicensis*, einem fakultativ intrazellulären Proteobakterium und opportunistischem Infektionserreger beim Menschen, entwickelt. Die Anwendung der beiden neuen Modelle gab uns die Möglichkeit die Pathogenese dieser Bakterien in Zebrafisch Larven zu untersuchen und die Merkmale zwischen einer durch ein obligat oder ein fakultativ intrazelluläres Pathogen verursachten Infektion zu vergleichen. Wir haben Virulenzeigenschaften beider Bakterien entdeckt und die Wirksamkeit von Antibiotika zur Behandlung einer Infektion in unserem Modell getestet. Ausserdem konnten wir durch die Verwendung transgener Zebrafisch-Linien die Reaktion des angeborenen Immunsystems auf eine Infektion mit beiden Pathogenen analysieren. Auf diese Weise haben wir zwei neue Infektionsmodelle zur Anwendung gebracht und das Zebrafisch-Modell als vielversprechendes und nützliches Instrument zu Untersuchung von bakteriellen Infektionskrankheiten bestätigt.

Chapter 1

General Introduction

1.1 Infectious Diseases

Infectious diseases are still among the major causes of death amid mankind (World Health Organisation, Fact Sheet N°310) and a permanent issue in food production among domestic animals and cultured plants. Moreover, wild animals are permanently exposed to pathogenic pressure and infectious diseases can pose a contributing factor to the reduction of populations and even extinction of whole species.

1.1.1 What is an infection?

An infection is the invasion and replication of an infectious agent inside a host organism. Infectious agents can be viruses, prions, bacteria, protozoa, fungi or parasitic worms. The immune system of the host can protect from or combat an infection.

1.1.2 Bacterial infections – The pathogenic life style

According to 16S rRNA sequence analyses of various terrestrial and aquatic habitats there exists an estimated number of 10^7 to 10^9 different bacterial species worldwide (Torsvik et al. 2002, Curtis et al. 2002, Schloss et al. 2004) of which fewer than 8000 have been described so far. The vast majority are believed to be non pathogenic compared to e.g. 518 bacterial species known as human pathogens (Cleaveland et al. 2001). Many bacteria are even vital symbionts of humans and other vertebrates and can account for up to 90 % of the total number of cells within an organism. Nevertheless, infections with bacterial pathogens can be life-threatening and are known to have the potential to cause fatal epidemics. But what makes a bacterium a pathogen? Bacteria are the oldest life forms we know and like every other species continuously adapting to their environment driven by the permanent force of evolution. Hence, some bacteria have adapted to utilise sunlight as energy source, some deep sea bacteria on the other hand have the ability to oxidise inorganic compounds as energy source and still others even lack major components of a functioning electron transport chain and are fully dependent on an eukaryotic host cell for the supply of energy and further nutrients. Bacteria that exclusively live and replicate inside a host cell are called obligate intracellular and comprise, for example, members of the Chlamydiaceae and Rickettsiaceae.

Bacteria that are able to replicate both inside and outside of a host cell are called facultative intracellular. While obligate intracellular bacteria are usually specialised pathogens, facultative intracellular bacteria can also be a ubiquitous part of the environment and only become pathogenic under certain circumstances, i.e. in immune suppressed individuals or newborns with an immature immune system. These bacteria are called opportunistic pathogens. Whence, it is not always possible to define a bacterium as a pathogen per se. Also more specialised facultative intracellular pathogens like *Salmonella spp*. thrive outside a host cell, but use their intracellular abilities when infecting a host to hide from the immune system, to actively cross physical barriers and spread inside the organism from cell to cell. Many obligate and facultative intracellular pathogens have developed abilities to survive and sometimes, even replicate inside professional phagocytes of the innate immune system like macrophages, an ability, maybe evolutionarily derived from infections in free-living amoebae which could be serving as a potential reservoir and training ground for these bacteria (Greub et al. 2004).

1.2 Animal models

Since pathogenic microbes were discovered as the cause of infectious disease, microbiologists and physicians were seeking to study their nature and pathogenesis under a scientific approach. *In vitro* studies, aiming to describe the morphology of agents as well as the pathology they cause in the host and *in vitro* experiments in cell culture are the most common methods to analyse infectious agents and the disease they cause. A further step of investigation was the development of animal models.

1.2.1 General features and applications

The first aim for the utilisation of animals as *in vivo* models for infectious diseases was to fulfill Koch's postulates for defining a pathogen as the definite causative agent of a disease. Later scientists discovered the power of animal models to study also the mechanisms of diseases *in vivo*. Since a host organism is the natural environment of a pathogen, only the study of an infection within such a host can give a holistic view of the infection process, including the reaction of the host immune system and strategies of the pathogen to overcome this. Also strategies for treatment like the use of anti-microbial drugs and their efficacy as well as possible adverse effects on the host can only be tried out within an *in vivo* model. A model organism used for these purposes should be well described in its anatomical, genetic and behavioural features. It should be possible to keep it ideally in large numbers and under laboratory conditions. Genetic tractability, a variety of

applied analytic methods and easy handling during experiments are further preferred features for a model organism. The most popular animal models cover more or less these requirements with having their individual strengths. The fruit fly *Drosophila melanogaster* for example is known for its easy genetic tractability. The nematode *Caenorhabditis elegans* is appreciated for its transparency and hence, easy microscopic accessibility. Both organisms can be kept in large numbers and are easily bred with short generation times. On the other side, these species are quite distantly related to vertebrates and therefore lack certain features like, for instance, a complex immune system. This problem is compensated with mammalian animal models like the house mouse *Mus musculus* and the common rat *Rattus norvegicus,* which on the other hand are inferior to the advantages of the invertebrate models through their smaller size, transparency or larger numbers of animals and a higher reproduction rate. This discrepancy increases with larger mammalian model animals like dogs, cats or even cattle. For these reasons a model organism, which could bridge this gap, is needed.

1.2.2 The zebrafish (*Danio rerio*)

Such an established and widely used model organism is the zebrafish *(Danio rerio).* First described by Francis Hamilton in 1822, the zebrafish was introduced to science by the US-American geneticist George Streisinger in the early 1970s. The small tropical freshwater fish, native to the southeastern Himalaya-region, provides numerous features that predestines it for research and contrast it favourably with other vertebrate animal models like mice. As a vertebrate, the zebrafish shares many similarities with mammals, including humans. Due to its small size and its undemanding nature concerning water parameters and food, it is easy and cost-effective to keep and to breed. With a reproduction rate of up to 300 eggs per clutch and a generation time of about 4 months, it is highly fertile. Because embryos are transparent and develop completely outside of the mother, it is easy to observe them under the microscope. The fast development and ruggedness of the embryos are further advantages. The genome of the zebrafish is fully sequenced and has been added to the Genome Reference Consortium (GRC) in 2010 following those of human and mouse. Additionally, it is amenable to both forward and reverse genetics and there is a wide and further increasing range of different mutant and transgenic strains available. Forward genetic screens combined with the high throughput potential make zebrafish a powerful model in functional genomics. A widely used technique to achieve a gene *knockdown* in embryos during the first days of development is done by injection of morpholinos (Nasevicius and Ekker, 2000). Commonly used techniques for reverse genetics in zebrafish are TILLING (Targeting Induced Local Lesions IN Genomes), a specific screening technique to detect certain *knockout* mutants (Moens et al., 2008) and the insertion of mutations at defined target sites in the genome

by specially designed Zinc-finger nucleases (Meng et al., 2008). Further recently applied techniques to modify and edit the zebrafish genome are done with TALENs (transcription activator-like effector nucleases) (Hwang et al. 2014) and the CRISPR/Cas system (Horvath & Barangou 2010, Blackburn et al. 2013, Hwang et al. 2013).

The zebrafish immune system displays many similarities to mammals, with counterparts for most of the human immune cell types (reviewed in Meeker et al. 2008). Molecular components, like Toll-like receptors (TLRs), a complement system as well as proinflammatory cytokines and acute-phase response proteins are also well conserved features of zebrafish immunity (Trede et al. 2004). The zebrafish innate immune system starts to develop as early as 24 hours post fertilisation (hpf) with primitive macrophages derived from the anterior lateral plate mesoderm (Herbomel et al. 1999), followed by neutrophils at 2 days post fertilisation (dpf) (Levraud et al. 2008). Zebrafish neutrophils have a similar morphology and function like mammalian neutrophils, including a polymorphic nucleus, primary and secondary granules, myeloid-specific peroxidase and a functional NADPH oxidase (Lieschke 2001, Brothers et al. 2011). Zebrafish neutrophils both phagocytose and kill bacteria (Colucci-Guyon et al. 2011) and are able to produce so called neutrophil extracellular traps (NETs) (Palic et al. 2007). The development of the adaptive immune system lags behind this. The first lymphocytes can be observed from 4 dpf, but the adaptive immunity takes up to 4 weeks to fully develop (Fig. 1). This gives the brilliant opportunity to observe exclusively the reaction of the innate immune system within an infection during the first week of larval development.

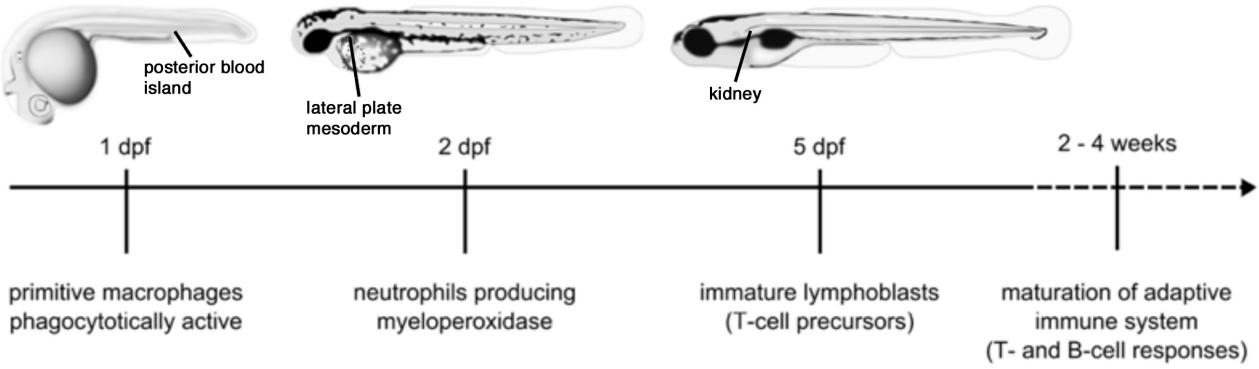

Figure 1 *Schematic overview of the development of the zebrafish immune system. Locations of myeloid haematopoiesis in the early zebrafish embryo are the posterior blood island and the lateral plate mesoderm close to the yolk sac. From 4 dpf granulopoiesis already starts in the kidney. Adapted from Meijer & Spaink, 2011.*

An important point in terms of infectious diseases is the ability of a pathogen to successfully conquer the different defensive lines of its respective host. Physical barriers, like skin and mucosa, are the first lines of defence, followed by tissue epithelia, cellular plasma membranes and of course the various cell types of the innate and adaptive immune system. Mechanical or chemical damage of the primary barriers initiates morphogenetic programs to rapidly restore tissue integrity and homeostasis. Pro-inflammatory molecular mediators recruit phagocytes (neutrophils and macrophages) to the site of the lesion to combat potentially invading pathogens. The extensive development of transgenic zebrafish strains, where innate immune cell populations are specifically labelled with fluorescent proteins and readily imaged in the transparent larva, gives the marvellous opportunity to visualise immune cell recruitment and inflammation *in vivo* (Hall et al. 2007/2009).

1.2.3 Development of an infection model in zebrafish larvae

As a model of infectious disease and immunity function the zebrafish becomes increasingly popular. The fact that most organ systems are functioning after 5 days post-fertilization (including all haematopoietic organs) and that almost all cell types of the human immune system have zebrafish counterparts, makes the zebrafish an ideal model for these purposes (Meeker and Trede, 2008). A variety of bacterial pathogens have already been tested for experiments in zebrafish (Kanther & Rawls, 2010). Until now, zebrafish have not been used to investigate infections of obligate intracellular bacteria like the Chlamydiae.

Certainly, it is not possible to infect any animal with just any kind of pathogen. Therefore, the first step in developing a model system is to test for the susceptibility of the model animal for the pathogen of interest. If the pathogen has not been described to cause disease in this animal species, a good alternative can be to develop the model by using a close relative of the pathogen that naturally infects the model animal. This has, for example, been very successfully achieved in the case of mycobacteria, where a zebrafish model was developed with the natural fish pathogen *Mycobacterium marinum* to investigate general features of mycobacteriosis that are also transferable onto infections with *Mycobacterium tuberculosis* in humans (Davis et al. 2002).
The second decision that has to be made is for the method of infection. The easiest way to infect the model animal is a controlled contamination of the environment or food with the pathogenic bacteria. However, this method is not always successful or results in a large variety between single experiments and is therefore lacking reproducibility. Hence, another possibility to develop an infection assay is to make use of an artificial infection procedure. These can comprise different methods like intravenous or intraperitoneal injections of a previously adjusted number of pathogenic bacteria or for example their direct administration into the respiratory tract. This method

also offers the possibility of penetrating primary physical and cellular barriers of the host, which may well be limiting for infections by a non-natural pathogen-host combination. After having defined the model animal, the pathogen and the infection method, the next step is to develop and apply tools to analyse the infection and evaluate the model. This includes for example quantification of the infection load, visualisation and localisation of the pathogenic bacteria, investigation of the pathological changes related to the infection and observation of the reaction of the host immune system. Once an assay has been developed and verified it can be applied to address particular questions by accordingly changing the parameters. For example, if genomic features of both the host and the pathogen are known, one could investigate the functions of certain virulence related genes of the pathogen or immunity related genes of the host by utilising mutant and transgenic strains of the model animal or the bacteria. Moreover, transgenic bacteria or certain cell types of the host that express fluorescent proteins enable the researcher to easily recognise the distribution of the bacteria inside the host organism, the behaviour of fluorescent tagged immune cells or preferred target cells for infection by the pathogen.

The set-up to perform microinjections in zebrafish requires somewhat more equipment. Firstly, a good binocular stereomicroscope with preferably two separate light paths for a three-dimensional view and a transmitted light base for more contrast is needed. For most injections performed in zebrafish embryos or larvae, a ten to fortyfold magnification is sufficient. Furthermore, a pressure based microinjection apparatus is needed to control the injection parameters like pressure and time pulse. The apparatus should keep a constant pressure throughout the experiment and the injection pulse should be adjustable in the milliseconds range. Commonly used devices are e.g. the FemtoJet from Eppendorff or the Pneumatic Pico Pump from World Precission Instruments (WPI). Finally, a micromanipulator is needed which serves as holder for the injection needle and is used to guide the needle to the injection site. For most microinjections into zebrafish larvae, a manual micromanipulator, which can be operated in a range between 10 and 100 μm, is sufficient. The anaesthetised and immobilised embryo can be injected at various sites, depending on the purpose. Injections into the musculature or simply into the yolk (Fig. 2D,G) can be used to cause a focal infection in a specific region. Injections into closed body cavities like the hindbrain ventricle or the otic capsule (Fig. 2C,E), which are usually devoid of immune cells, can be used to observe specifically immune cell recruitment. The injection into the notochord (Fig. 2F) can cause a focal infection, which is virtually inaccessible to phagocytes. A systemic infection can be achieved by intravenous injection into the caudal vein either via the posterior blood island (Fig. 2A) or the Duct of Cuvier (Fig. 2B).

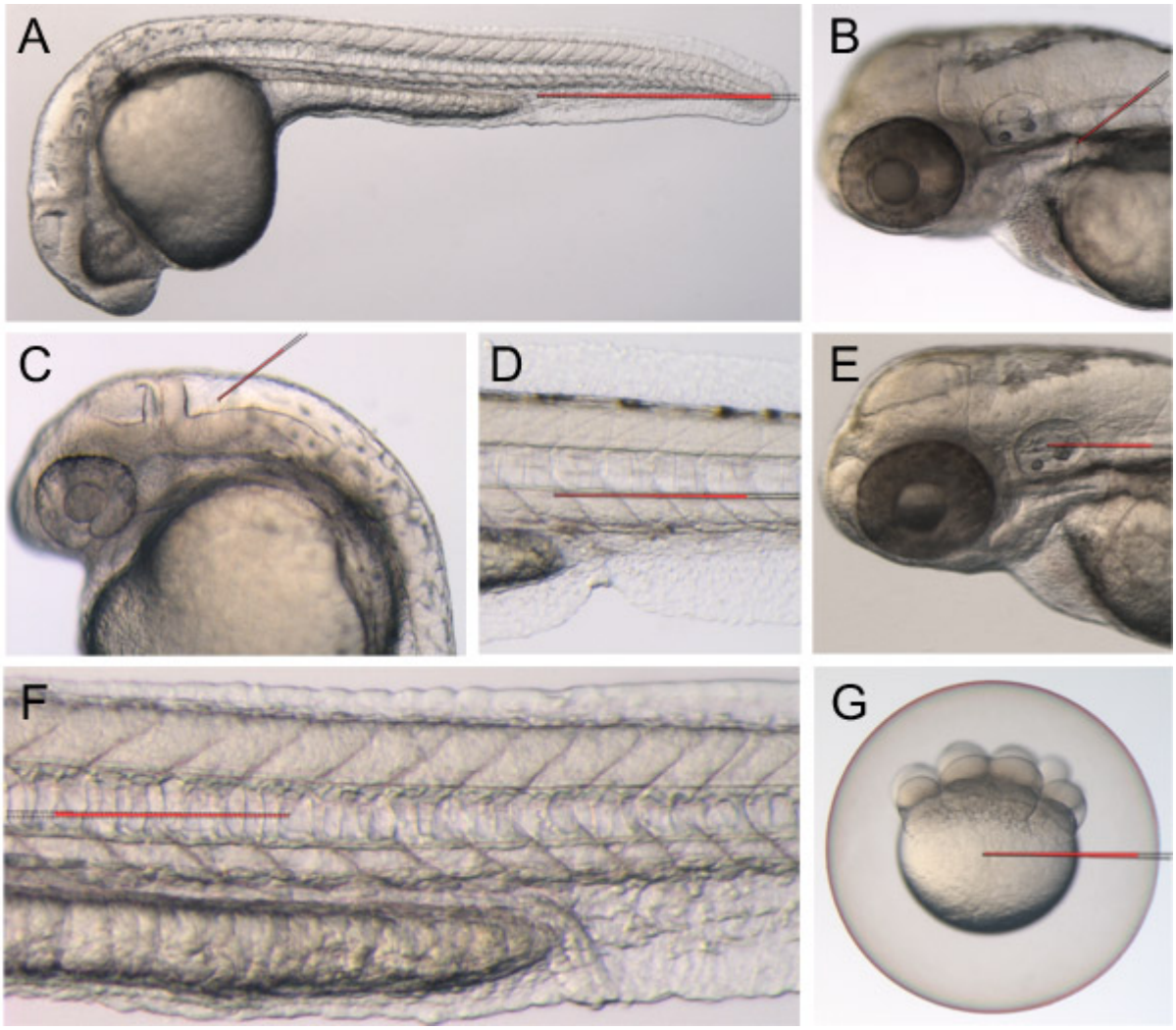

Figure 2 *Variety of injection sites of the zebrafish embryo. A systemic infection can be achieved by intravenous injection either into the posterior blood island (A) or the Duct of Cuvier (B). Focal infections can be attained by injection into the hindbrain ventricle (C), intramuscular (D), into the otic vesicle (E), into the notochord (F) or into the yolk sac (G). Adapted from Benard et al. 2012.*

The infection procedure can be practised and observed by using stained or fluorescent fluids, which also can be added to the bacterial suspension. The use of fluorescence tagged dextran for example allows monitoring of the exact distribution of the injected fluid inside the larval organism (Figure 3).

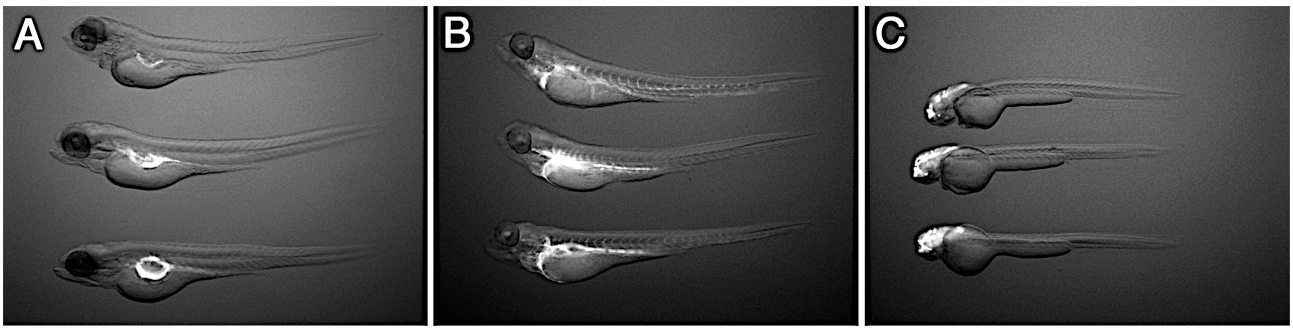

Figure 3 *Injection of Dextran 70kD conjugated to TexasRed (Invitrogen) into the swim bladder of 4 dpf (A), intravenously into 3 dpf (B) and into the brain ventricle of 2 dpf (C) old larvae. The 70kD Dextran is sufficiently large molecule so as not to be able to traverse epithelial and endothelial borders and is therefore perfectly suitable to observe the target and distribution of the injected solution.*

1.2.4 Bacterial diseases in fish

Fish can be infected by a large variety of bacterial pathogens, which is of interest on it's own but also raises the possibility of using fish to investigate mechanisms of infection for the same or closely related pathogens of humans. Many of them are not specific just for a single host species and can also occur worldwide (Roberts, Fish Pathology, 4th edition, 2012). Well-known Gram-negative pathogens are e.g. *Flavobacterium columnare* causing columnaris disease, *F. psychrophilum* causing cold water disease and *F. branchiophilum* and *Tenacibaculum maritimum* (former *Flexibacter maritimus*) which both can cause bacterial gill disease and fin rot. Gram-negative facultative anaerobic pathogens comprise enterobacteria like *Edwardsiella tarda* causing Edwardsiella septicaemia, *Edwardsiella ictaluri* causing enteric septicaemia of catfish and *Yersinia ruckeri*, the causative agent of enteric red-mouth (ERM). Further facultative anaerobic Gram-negative pathogens are several *Vibrio spp.* like *V. anguillarum* causing vibriosis and septicaemia and different *Aeromonas spp.* causing furunculosis and septicaemia as well. Among Gram-positive pathogens are e.g. *Renibacterium salmoninarum,* the causative agent of bacterial kidney disease (BKD) in salmonids and *Lactococcus garviae* and several *Streptococcus spp.,* which can cause septicaemia. Important acid-fast pathogens of fish are mycobacteria like *Mycobacterium marinum* which cause mycobacteriosis. Obligate intracellular Gram-negative pathogens of fish comprise e.g. *Rickettsia salmonis* causing strawberry disease and the quite diverse group of chlamydial pathogens that cause epitheliocystis in a broad range of fishes. Taken together, the question arises as to whether fish could serve as intermediate hosts for zoonotic transmission and furthermore, whether model species such as zebrafish could be utilised as a genetically tractable model for chlamydial infections?

1.3 Bacterial pathogens studied in this thesis

This is divided into naturally occurring bacterial pathogens with the focus on suspected novel chlamydial pathogens thought to cause the disease epitheliocystis in fish and secondly, to investigate whether zebrafish can be used as a model for the chlamydial pathogen, *Waddlia chondrophila*, an obligate intracellular bacterium and how this might compare with a facultative intracellular bacterium, *Cronobacter spp*. Both these bacteria are able to infect macrophages and epithelial cells, so the transparent zebrafish model should potentially enable us to determine which of these cell types is preferentially targeted *in vivo*.

1.3.1 Epitheliocystis agents

Epitheliocystis is a bacterial infectious disease of the gills of juvenile and adult and of developing gills, skin and fins of larval fish (Fig. 4). The pathologic condition was first described in common carp (*Cyprinus carpio*) by the German scientist Marianne Plehn in 1920 (Plehn, 1920) and almost half a century later further characterised and named by Hoffman et al. in a case of infected bluegills (*Lepomis macrochirus*) (Hoffman et al. 1969). Since then the disease has been diagnosed in many more different wild and farmed species of freshwater and marine fishes worldwide, covering their whole diversity from carps (Kumar et al. 2012) and salmonids (Draghi et al. 2004) over sea bass (Anderson and Prior 1992), sea bream (Crespo et al. 1999) and sea dragon (Langdon et al. 1991), a relative of seahorses and pipefish, up to evolutionary ancient species like sturgeons (Groff et al. 1996) and sharks (Polkinghorne et al. 2010). The prevalence of epitheliocystis is generally greater in cultured fish than in wild fish (Novak and LaPatra, 2006).

Main hallmarks of the disease are small to large inclusions of basophilic granular material, representing bacteria, within hypertrophied epithelial cells of the gills or skin or fins of infected animals. Some studies in Atlantic salmon have identified lamellar epithelial cells as the preferred target (Nowak and Clark, 1999). The number, size and distribution of the cysts vary with different cases of infection and also between individual infected fish. Many epitheliocystis cases, especially in wild fish, are often described without severe pathology or clinical signs, implying that in these cases the host immune system is able to limit the levels of infection. In benign infections, a cyst can be enveloped by a layer of squamous or cuboidal epithelium, but no further response of the host is observed. On the other hand, an infection can also induce a strong host response, including proliferation of affected host epithelia and excessive mucus production, sometimes referred to as hyperinfection (Mitchell and Roger, 2011). Pathological changes can include multifocal diffuse epithelial hyperplasia, lamellar filling, lamellar fusion, bridging across tips of adjacent lamellae, focal necrosis of epithelial cells and infiltrates of macrophages. In infections causing such a

proliferative host response, affected fish have been described as lethargic and displaying signs of respiratory distress, such as increased ventilation and gasping at the water surface. Deformation of the opercular cover, increased mucus production and distortion of the lamellar structure have also been described (Mitchell and Roger, 2011). The mechanism that triggers a proliferative response is still unknown. Age and immune status of the fish, as well as stress or other environmental parameters have been proposed as possible factors. Mortality due to epitheliocystis is usually associated with the proliferative form of the disease and is much higher in juvenile and larval fish with mortalities up to 100 % (Katharios et al. 2008), compared to adult fish.

The causative agents, identified by PCR, in situ hybridisation, immuno-histochemistry and electron microscopy, are all Gram-negative bacteria mainly of the phylum Chlamydiae. First described were *Ca.* Piscichlamydia salmonis identified in farmed marine Atlantic salmon (*Salmo salar*) (Draghi et al. 2004) and *Ca.* Clavochlamydia salmonicola identified in farmed freshwater Atlantic salmon and wild brown trout (*Salmo trutta*) (Karlsen et al. 2008). Both species were later found to also cause mixed infections in Swiss wild brown trout (Schmidt-Posthaus et al. 2011). More recently, non-chlamydial epitheliocystis agents belonging to the proteobacteria have been discovered. The beta-proteobacterium *Ca.* Branchiomonas cysticola was identified in farmed marine Atlantic salmon (Toenshoff et al. 2012) and the gamma-proteobacterium *Endozoicomonas elysicola* was found as an agent within cobia (*Rachycentrum canadum*) (Mendoza et al. 2013). Despite numerous attempts, culturing of an epitheliocystis agent has not succeeded so far, posing a major obstacle in developing a model system to study the disease. The weight of our investigations, including those forming part of this thesis, has been to develop means to study the morphology and molecular biology of epitheliocystis agents *in situ* and failing success in cultivation, explore potential infection strategies using a non-model fish host with known susceptibitly to epitheliocystis (Fig. 4).

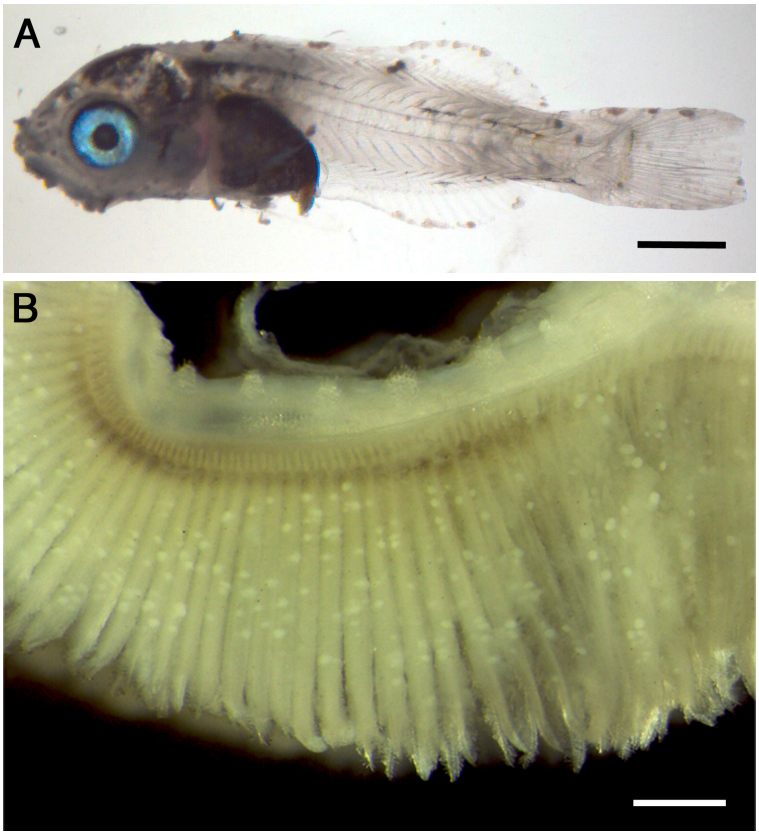

Figure 4 *Light microscope appearance of epitheliocystis in wet-mounted preparations of a 24 dpf old Diplodus puntazzo larva (A) and a dissected single gill arch of a juvenile Sparus aurata (B). Bacterial inclusions appear as brownish or whitish spots on the skin and fins of the larval (A) and on the gill lamellae of the juvenile seabream (B). Scale bars 1 mm.*

1.3.2 *Waddlia chondrophila*

The aquatic chlamydial pathogens of fish have also numerous terrestrial relatives, which are pathogens of land vertebrates including humans. So far all known members of the phylum Chlamydiae are obligate intracellular pathogens with a large variety of hosts within the whole animal kingdom. The first discovered family was the *Chlamydiaceae*, classical pathogens of humans and animals. During the past two decades more and more new families and species were discovered, dramatically increasing the size of the phylum (Collingro et al. 2011). Up to date eight different families have been recognised, although a recent metagenomics environmental study proposed a vastly greater 181 families (Lagkouvados et al. 2014). One of the better described families is the *Waddliaceae*. It is currently composed of the two species *Waddlia chondrophila* (Dilbeck et al. 1990, Henning et al. 2002) isolated from cattle and *Waddlia malaysiensis* isolated from fruit bats (Chua et al. 2005), with *W. chondrophila* giving the family its name.

W. chondrophila is a pathogen of cattle and was first isolated from a cow abortion in the United States (Dilbeck et al. 1990) and later again from a similar case in Germany (Henning et al. 2002). Members of the Chlamydiae are known for their high zoonotic potential and unspecific infectivity across species borders. One of the oldest and best-characterised examples is *Chlamydia psittaci*,

the agent of psittacosis in birds and humans (Vanrampay et al., 2007) or *Chlamydia abortus*, an abortive agent of small ruminants and humans (Thomson et al. 2005; Voigt A, et al. 2012; Knittler et al., 2014). Indeed, also Waddlia chondrophila has been found to be of considerable zoonotic risk when shown by serological tests and quantitative real-time PCR in cases of human miscarriage and respiratory disease (Baud et al. 2007, 2011; Haider et al. 2008; Hornung et al. 2014).

The genome of *W. chondrophila* has been sequenced from both isolates. It consists of a 2.1 Mb chromosome (Bertelli et al. 2010). This is twofold larger compared to classical chlamydial genome sizes which are usually around 1 Mb and is closer to the size of a genome of Verrucomicrobia, the next free-living relatives of the Chlamydiae, with a genome size of about 2.3 Mb (Hou et al. 2008). The *W. chondrophila* genome encodes for all necessary components for a complete citric acid cycle and glycolysis with a subsequent electron transport chain. Furthermore, it enables the production of a F1F0 ATP synthase complex for an increased ATP synthesis. *W. chondrophila* is able to produce ten different amino acids and all nucleic acids from imported intermediary metabolites. Moreover it possesses a variety of enzymes for an enlarged lipid metabolism (Bertelli et al. 2010). These features make *W. chondrophila* more independent of its host and more flexible to environmental influences compared to other classical members of the family *Chlamydiacea*. In addition to the circular chromosome, a 15.6 Kb plasmid was discovered in one of the two isolates (WSU 86-1044) encoding for 22 different proteins. In the second isolate (2032/99) 9 of these protein encoding genes can be found in the chromosome indicating an integration of the plasmid into the chromosome in this strain (Collingro et al. 2011).

W. chondrophila displays a classical chlamydial biphasic life cycle with elementary bodies (EBs) and reticulate bodies (RBs). The metabolically almost inactive EBs are the infectious stage. They are characterised by their small size (0.4 μm), a strongly condensed chromosome and a highly cross-linked outer protein layer. Major components of this coating are proteins of the Omc, Omp and Pmp families, many of them with still unknown function and so far unique for *W. chondrophila*. Proteins of this layer are likely involved into attachment and internalisation. Additionally *W. chondrophila* possesses a Type III secretion system (T3SS), which also could be involved in internalisation and subsequent steps of the infection by injection of bacterial effectors into the host cell (Bertelli et al. 2010). After attachment and uptake of the *W. chondrophila* EB into the host cell a so called bacterial-containing vacuole (BCV) is formed. Immediately after uptake, host cell mitochondria are rapidly recruited and form an intimate association with the BCV after which parts of the Endoplasmic Reticulum (ER) are recruited to form a complex, which is usually located in a perinuclear fashion. During this process, the EB releases its protein coat and transforms into the replicating RB form. RBs divide by binary fission and are often in direct contact with mitochondrial membranes forming the typical *W. chondrophila* inclusion. After several rounds of replication, RBs

re-transform into EBs and are released into the environment after lysis of the host cell to start a new cycle of infection (Kocan et al. 1990, Croxatto et al. 2010). *W. chondrophila* can also form aberrant bodies (ABs) which are considered as the persistent stage of the Chlamydiae (Kebbi-Beghdadi et al. 2011).

As an obligate intracellular pathogen, *W. chondrophila* replication is always dependent on an appropriate host cell that can be successfully infected by the EB. The first cultivation of *W. chondrophila* was done in bovine turbinate cells and mouse macrophages (Dilbeck et al. 1990, Kocan et al. 1990). Subsequent in vitro studies showed that *W. chondrophila* is furthermore able to infect and replicate in McCoy cells, buffalo green monkey cells, human fibroblasts (Henning et al. 2002), Vero cells, human pneumocytes and endometrial cells (Kebbi-Beghdadi et al. 2011), as well as in human macrophages (Goy et al. 2008). During the infection of macrophages *W. chondrophila* avoids its degradation by successfully preventing the fusion of the endosome with a lysosome (Croxatto et al. 2010) (Fig. 5).

Moreover, it was found that freshwater amoebae of the genus *Acanthamoeba* spec. are susceptible to infection with *W. chondrophila* (Lamoth et al. 2010). A recent study showed that *W. chondrophila* is also able to enter and proliferate in the two fish cell lines EPC-175 and RTG-2, the former isolated from fathead minnow (*Pimephales promelas*) and the latter from rainbow trout (*Oncorhynchus mykiss*) (Kebbi-Beghdadi et al. 2011). Concerning the environmental distribution of *W. chondrophila*, it was not only found to be present in its known mammalian hosts but indeed also in aquatic environments diverse as sediments from the eastern Mediterranean Sea (Polymenakou et al. 2005) as well as in freshwater samples from well water sources in Spain (Codony et al. 2012). According to these findings, it was postulated that freshwater protists and fish could potentially serve as an aquatic reservoir for *W. chondrophila* and one possible transmission route is water-borne.

Taking these findings together, it would appear that *W. chondrophila* could be a worthwhile starting point for investigating zebrafish as a possible model host. We also have reagents for detecting *W. chondrophila* in larvae and as well, we have transgenic zebrafish which will help answer the question as to whether *W. chondrophila* is able to utilise or avoid destruction by the innate immune cells during the early stages of infection. Should this be successful, this would be the first zebrafish model for a member of the Chlamydiae.

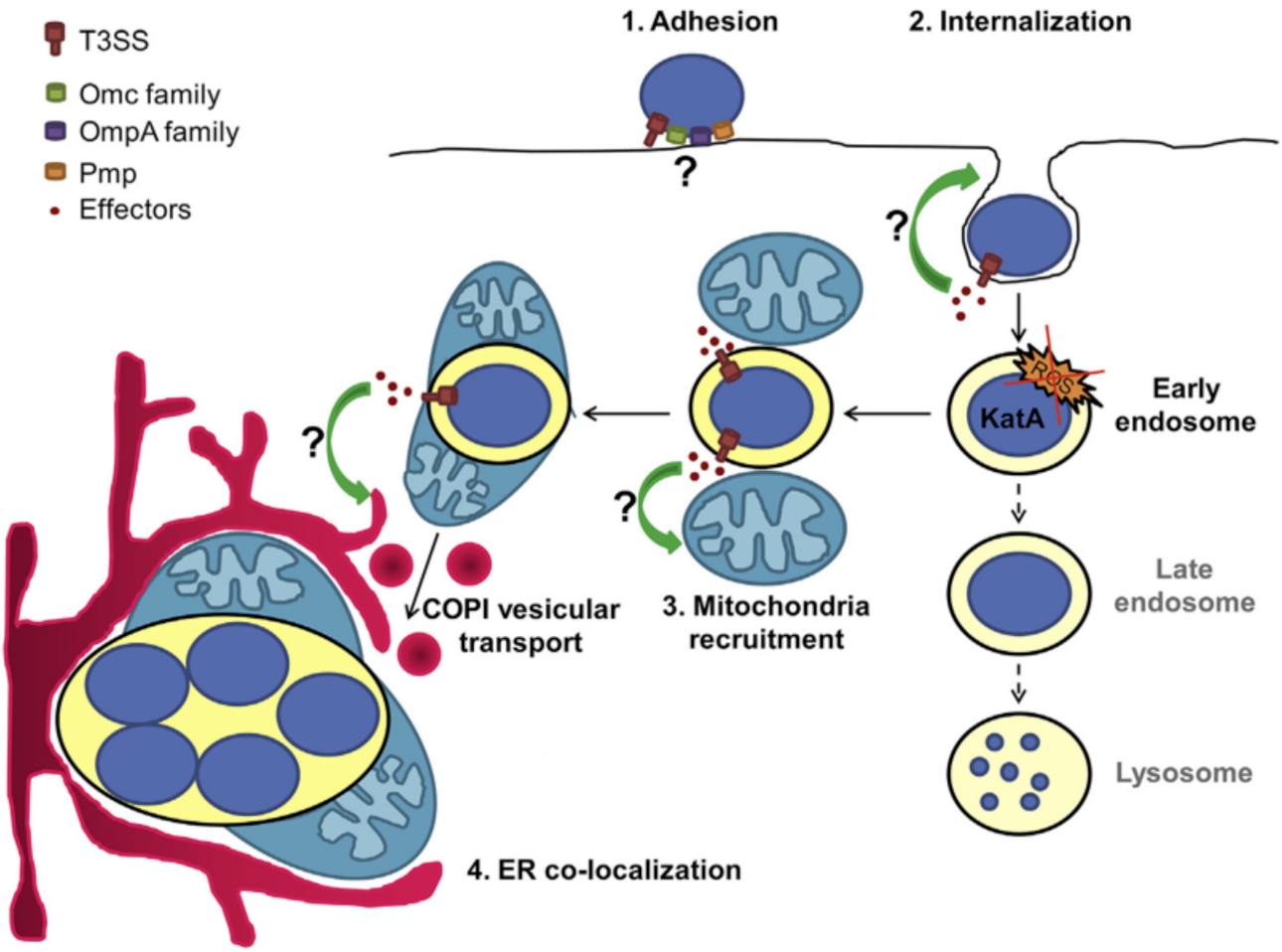

Figure 5 *Scheme of Waddlia infection within macrophages. Adapted from de Barsy & Greub 2013.*

1.3.3 *Cronobacter spp.*

Cronobacter is a newly described genus of Gram-negative bacteria belonging to the familiy of Enterobacteriacea. Other members of this family are typical commensuals like *Escherichia coli*, but also the causative agent of the Black Death *Yersinia pestis* and pathogens like *Salmonella spp.* and *Shigella spp.*. The novel genus *Cronobacter* originates from the former genus *Enterobacter* (Iverson et al. 2008). Up until now, the *Cronobacter* genus consists of seven species, which are *Cronobacter sakazakii, C. malonaticus, C. muytjensii, C. turicensis, C. dublinensis, C. universalis* and *C. condimenti* (Jaradat et al. 2014).

Cronobacter spp. are opportunistic pathogens and with exception of *C. condimenti*, all members of the genus have been linked to infections in humans (Cruz-Córdova et al. 2012). Infections with *Cronobacter* can cause meningitis, necrotising enterocolitis (NEC), bacteraemia and sepsis (Healy et al. 2010). Newborn, infants, immunocompromised and elder people are particularly at risk. Nosocomial infection of infants with Cronobacter via contaminated powdered infant formula has been identified as a possible epidemiological route. Although infections in neonates occur rarely,

these cases are often quite severe with a fatality rate of up to 80 % (Healy et al. 2010). Compared to other enterobacteria *Cronobacter spp.* possesses a high tolerance against heat, drought and acidic conditions and furthermore is able to attach to and form biofilms on plastics, glass, metals and even Teflon (Lehner et al. 2005), which physically protects the bacteria from environmental stresses and during an infection also from the host immune system. *Cronobacter spp.* is ubiquitous in the environment and can be found in soil, freshwater and is often associated with plants. Accordingly, it has been isolated from a large variety of foods and even household vaccuum cleaner dust (Table 1), all of which are further possible sources of contamination. One very likely entry point into humans is the gut mucosa where *Cronobacter spp.* can also cause NEC (Liu et al. 2012). Once *Cronobacter spp.* has entered the host systemically, it can distribute in the blood circulation, causing bacteraemia or sepsis. With a tropism towards the central nervous system, *Cronobacter spp.* can also cross the blood-brain barrier causing meningitis (Healy et al. 2010). The genome of *C. turicensis* contains 223 putative virulence genes (Stephan et al. 2011). One more feature of *Cronobacter* is its ability of to survive and persist within human macrophages (Townsend et al. 2007). The role and interaction of single virulence factors during the whole pathogenesis remains still elusive and more virulence and disease related genes are likely to be discovered. Although mouse models have been used to investigate the disease progression, there is a need for an animal model system with which to readily test the wide range of putative virulence genes, preferably in a high throughput manner. The zebrafish is one such possibility and as a first step, we set out to test its feasibility as a *Cronobacter* host.

Food/environment
Infant food, beverages and processed foods
Infant food
Follow-up formula
Rice flour
Chocolate and cakes, vanilla cream bars
Herbal tea, iced tea
Weaning foods
Rice, corn, soy, grain, starches, potato flour, pasta, cereals
Tofu, sodium casinate
Plants and spices
Liquorice, thyme, anise, chamomile, fennel, sage
Mixed spices
Attieke, barley, biscuits, cereals, cowpea paste, dry nuts, grains, herbs and spices, red algae, sorghum, peas
Dried herbs Mixed spices, black pepper and white pepper, curry and mixed herbs
Oats, barley, wheat
Dried vegetables
Fresh produce
Salads, and herbs

Mixed vegetables, salad Sprouts, fresh herbs and salads, parsley, dill, coriander, celery, basil
Animal products Camel, eggs, cheese, milk, pork, fish and products, poultry, sausages, shellfish, shrimp Beef and products, pork, burgers, minced meat from beef and pork
Environment Milk powder and environment Water, soil and grass Household vacuum dust

Table 1 *Foods and environments from which Cronobacter was isolated. Adapted from Jaradat et al. 2014.*

1.4 Topics of the thesis

Knowledge about infectious diseases in fish and the biology of the causative agents is often still narrow. Since epitheliocystis affects all kinds of fish species worldwide and is considered as an emerging disease of aquaculture, one objective of this study was the investigation of this infectious gill disease in different wild and farmed fish species. The central aim was the identification and description of the causative agents in discovered cases of naturally occurring infections and outbreaks in aquaculture farms, by analysing gill samples of infected fish by PCR amplification, cloning and sequencing of the 16S rRNA sequence to reveal phylogenetic relationships of the bacteria and fluorescence in situ hybridisation (FISH) for their detection inside the lesions. The pathology of the infection was described by standard histological examination techniques, including stained histological sections and by transmission electron microscopy (TEM), which also served to examine the ultrastructure of the bacteria and inclusions. Further three-dimensional ultrastructural analyses were performed by focused ion beam scanning electron microscopy (FIB-SEM).

Given the wide variety of bacterial agents causing disease in fish, implicates the opportunity to utilise fish as a model organism to study basic mechanisms of pathogenesis and immune function. Therefore the second aim of this work was the establishment of a zebrafish *in vivo* model for infections with intracellular bacteria. For this purpose different methods to infect larval zebrafish, like bath-immersion and microinjection, were carried out to determine the pathogenicity of different intracellular pathogens towards the larvae. After having settled a defined infection assay and variable methods to analyse the infection, like immunofluorescence staining, confocal microscopy, quantitative PCR and electron microscopy, further experiments were applied to the model to target questions concerning the pathogenicity of the bacteria or the immune response of the host.

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World Health Organisation (WHO), Fact Sheet N°310

Chapter 2

Candidatus Syngnamydia Venezia, a Novel Member of the Phylum Chlamydiae from the Broad Nosed Pipefish, Syngnathus typhle

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Article published in PloS ONE

Personal contribution:

Planning the experiments, dissection of gills and isolation of inclusions, DNA extraction, PCR amplification and cloning, sequence analysis, fluorescence in situ hybridisation, confocal microscopy and image analysis, preparation of the figures, writing of the manuscript

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Candidatus Syngnamydia Venezia, a Novel Member of the Phylum *Chlamydiae* from the Broad Nosed Pipefish, *Syngnathus typhle*

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Abstract

Chlamydia are obligate intracellular bacteria and important pathogens of humans and animals. *Chlamydia*-related bacteria are also major fish pathogens, infecting epithelial cells of the gills and skin to cause the disease epitheliocystis. Given the wide distribution, ancient origins and spectacular diversity of bony fishes, this group offers a rich resource for the identification and isolation of novel *Chlamydia*. The broad-nosed pipefish (*Syngnathus typhle*) is a widely distributed and genetically diverse temperate fish species, susceptible to epitheliocystis across much of its range. We describe here a new bacterial species, *Candidatus* Syngnamydia venezia; epitheliocystis agent of *S. typhle* and close relative to other chlamydial pathogens which are known to infect diverse hosts ranging from invertebrates to humans.

Citation: Fehr A, Walther E, Schmidt-Posthaus H, Nufer L, Wilson A, et al. (2013) *Candidatus* Syngnamydia Venezia, a Novel Member of the Phylum *Chlamydiae* from the Broad Nosed Pipefish, *Syngnathus typhle*. PLoS ONE 8(8): e70853. doi:10.1371/journal.pone.0070853

Editor: Deborah Dean, University of California, San Francisco, University of California, Berkeley, and the Children's Hospital Oakland Research Institute, United States of America

Received November 22, 2012; **Accepted** June 27, 2013; **Published** August 12, 2013

Funding: This study was supported by SBF Project C09.0039 to L. Vaughan as part of COST Action 867: Fish Welfare in European Aquaculture. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Host-pathogen co-evolution is an evolutionary arms race driving the simultaneous evolution of pathogen virulence and host defenses. This interplay is especially tight between obligate intracellular microbes, such as members of the phylum *Chlamydiae*, and their eukaryotic hosts. The majority of empirical data on this phylum comes from studies of a single family, the *Chlamydiaceae*, whose members are major pathogens of terrestrial vertebrates, including humans. Due to their obligate intracellular biphasic lifestyle, *Chlamydia* cycle between an infectious particle, the elementary body (EB), and vegetatively replicating bodies (RBs) undergoing clonal expansion in specialised membrane-bound cytoplasmic organelles or inclusions. These features impose tight constraints on chlamydial evolution, which appears to be more influenced by recombination when different inclusions within a cell fuse, rather than by genetic exchange with other free-living bacteria [1].

Although molecular clock approaches have not been applied to the study of chlamydial evolution, the rate of genetic change in this group appears to be slow, given a high degree of synteny between different human strains of the species *Chlamydia trachomatis* or *Chlamydia pneumoniae* [1,2,3]. The evolutionary origins of the phylum *Chlamydiae* are old, associated with the rise of the cyanobacteria and plant life. However, it remains unclear to what extent the features of the family *Chlamydiaceae* are characteristic for the phylum as a whole. Answers are beginning to come through genomic studies into members of other families, notably the *Waddliaceae* [4], *Protochlamydiaceae* [5,6], *Parachlamydiaceae* [7], *Criblamydiaceae* [8,9] and most recently the *Simkaniaceae* [10]. Their genomes are intermediate in size (2.1–2.6 Mbp) between the *Chlamydiaceae* (0.9 Mbp–1.2 Mbp) and free-living bacteria such as *E. coli* (4.6 Mbp), but are comparable in size to the closest free-living relatives in the phylum *Verrucomicrobia* [11].

There appears to be a core set of genes shared by the phylum *Chlamydiae* [10], including cysteine-containing periplasmic and membrane proteins present in families known to be pathogens of land vertebrates (*Chlamydiaceae*, *Waddliaceae*, *Parachlamydiaceae* and *Simkaniaceae*), and which, at least in the *Chlamydiaceae*, are thought to be essential for maintaining membrane structural integrity in the absence of a peptidoglycan layer [12]. Some of these proteins are also associated with responses of the bacteria to a host humoral immune response [13], which could be a feature separating primary vertebrate chlamydial pathogens from chlamydial pathogens of unicellular organisms. In recent years, many novel members of the phylum *Chlamydiae* have been isolated from environmental aqueous sources using the highly successful amoebal co-culture method, leading to an inevitable bias in our understanding of their function. Indeed, there is a school of thought which proposes that free living amoebae may have provided an initial training ground for evolution of *Chlamydiae*, prior to their expansion into multicellular hosts and finally land-based animals and birds [7,14,15]. If this is the case, the question is who were the intermediate evolutionary hosts? Given the deep

evolutionary history of *Chlamydia*, it follows that the major vertebrate hosts of the *Chlamydiae* prior to the emergence of land animals were fish.

The major chlamydial disease of fish is epitheliocystis, a term first coined by Hoffmann and colleagues [16] to describe infections of bluegill sunfish (*Lepomis macrochirus*) by bacteria ascribed to the "psittacosis-lymphogranuloma-trachoma group (*Chlamydozoaceae*)" based on appearance in transmission electron microscopy (TEM). The primary targets of this disease are epithelial cells of the gills and the skin, with the perinuclear inclusions forming cysts ranging in size from a few micrometers to many tens of micrometers. Epitheliocystis has been described in more than 50 fish species [17,18,19], with a wide range of morphologies infecting both juveniles and adults. Molecular data has only recently become available [20,21,22,23,24] and in only a few cases do we have both sequence information and ultrastructural data for infectious strains [20,22,24,25]. This lack of information lies at the core of debates concerning the assignment of different ultrastructural morphologies to different chlamydial species and their putative developmental stages, a discussion that can only be resolved by a considerable expansion of our coverage of epitheliocystis, now further underlined by a recent report suggesting that intracellular bacteria other than members of the *Chlamydiae* can also cause epitheliocystis [26]. In addition, apart from *Ca.* Clavochlamydia salmonicola [22,24], we have rarely found unequivocal morphological evidence for elementary bodies in epitheliocystis inclusions. This raises the question at what stage the biphasic life cycle, a central feature of the family *Chlamydiaceae* and for a long time part of the dogma of what "makes a *Chlamydia* a *Chlamydia*", arose in the evolution of the phylum [18,19,24]. To explore such possibilities in greater depth, suitable marine vertebrate models of *Chlamydia* infection are required to facilitate the investigation of host-pathogen evolution.

Pipefish and seahorses of the *Syngnathidae* are the focus of evolutionary studies at both the genetic and behavioural levels in many parts of the world [27,28,29]. This research often involves the collection of fish from free-living populations, requiring basic data on their population genetic structure. The European broad-nosed pipefish (*Syngnathus typhle*) is one case in point, with a pan-European distribution of genetically distinct populations [30]. Northern populations of this species arose after the end of the last glacial maxima (ca. 20,000 years ago) and contemporary populations of this species are exposed to very different environmental conditions. Given the clear genetic differences among populations of *S. typhle* [30], their pathogens might be expected to show a corresponding genetic diversity, reflecting an extended period of host-pathogen coevolution. Pipefish are susceptible to various diseases [31], including epitheliocystis (Schmid-Posthaus, personal observations). As a first step towards establishing whether chlamydial infections in pipefish could offer an appropriate evolutionary model, we have begun the characterisation of epitheliocystis in pipefish collected from the Lagoon of Venice, one of the most genetically diverse populations of *S. typhle* [30]. The epitheliocystis agent we describe below has a number of attractive features, which have the potential to make it a model for *Chlamydia* research.

Materials and Methods

Sample Collection

S. typhle were collected by trawling (4 mm mesh) eelgrass beds in the Lagoon of Venice (45°13.86′N, 12° 16.59′E) in June and September 2011 and transported to the laboratory in 60 l tanks, with constant aeration. Animals were euthanized in buffered 3-aminobenzoic acid ethyl ester (MS 222®, Argent Chemical Laboratories, Redmont, USA) in filtered seawater and immediately dissected. Tissue samples for histopathological analysis were fixed in 10% buffered formalin in seawater. Gills were removed under aseptic conditions and examined under a dissecting microscope for lesions, including the presence of epitheliocystis. When present, cysts were dissected free of surrounding fine gill lamellar tissue, rinsed in several changes of sterile sea water and prepared directly for DNA extraction or immediately frozen in liquid nitrogen for later analysis. Gill lamellae from fish containing cysts were also fixed in 10% buffered formalin for histology or in 2.5% gluteraldehyde in 0.1 M sodium phosphate buffer, pH 7.5 for electron microscopy. Formalin-fixed gill samples were paraffin-embedded and sections of 3 µm thicknesswere stained with haematoxylin-eosin (H&E) for histopathological examination.

DNA Extraction, PCR Amplification and DNA Sequencing of Epitheliocystis Positive Gills and Isolated Cysts

Genomic DNA was extracted from fresh or frozen gill tissue or from individual cysts using a commercial DNA extraction kit, according to the manufacturer's instructions (DNeasy Tissue kit; Qiagen, Hilden, Germany). The presence of chlamydial DNA in the samples was subsequently determined by broad-range order *Chlamydiales*-specific 16S rRNA PCR, targeting the *Chlamydiales*-specific 280 bp 16S rRNA gene signature sequence [23] or the nearly full length 16S rRNA gene of 1500 bp, as previously described [20]. Negative controls (dH_2O) were performed in triplicate. PCR products were visualised by UV transillumination (254 nm) following the separation of PCR products by agarose gel electrophoresis.

Freshly-amplified 16S rRNA gene PCR products were ligated into the pCR2.1-TOPO cloning vector (Invitrogen, Basel, Switzerland) and transformed into *E. coli* TOP10 chemically-competent cells (Invitrogen), according to the manufacturer's instructions. 5–10 individual clones derived from a single cyst or from gill lamella containing multiple cysts were screened by restriction digestion and inserts were capillary sequenced on a 3130xl Genetic Analyzer (Applied Biosystems) using M13 forward (5′-CGCCAGGGTTTTCCCAGTCACGA-3′) and M13 reverse primers (5′-AGCGGATAACAATTTCACACAGGA-3′).

Bioinformatics

Sequences were analysed with CLC Main workbench, version 6.8.1 (CLC bio Denmark), trimming and editing sequences after reviewing base-calls for accuracy. After alignments with ClustalW2 [32] or MUSCLE [33] on phylogeny.fr [34] using default settings, fasta files were generated. To examine the relationships between the sequences, phylogenies were constructed in MEGA5 [35]. Phylogenetic trees were inferred by the neighbour-joining (NJ) and the maximum-parsimony (MP) methods and tree reliabilities were assessed by 1000 bootstrap replicates [36]. Sequences were also identified using Blastn searches against the nucleotide collection of GenBank (www.ncbi.nlm.nih.gov) [37].

Transmission Electron Microscopy (TEM)

Gill branches fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.5 at 4°C were prepared for embedding into epoxy resin and for TEM according to standard procedures. Gill sections containing epitheliocystis lesions were selected from epoxy resin blocks using semithin sections (1 µm) stained with toluidine blue (Sigma–Aldrich). Ultrathin sections (80 nm) were mounted on copper grids (Merck Eurolab AG, Dietlikon, Switzerland), contrasted with uranyl acetate dihydrate (Sigma–

Aldrich) and lead citrate (Merck Eurolab AG) and investigated using a Philips CM10 transmission electron microscope. Images were processed with Imaris (Bitplane, Zurich) and assembled for publication using Adobe Photoshop.

Fluorescence In Situ Hybridisation (FISH)

E. coli TOP10 transformed with the full-length 1500 bp 16S rRNA gene fragment of fragments of *Waddlia chondrophila* [4] or *Ca.* Syngnamydia venezia (the major consensus amplicon, GenBank Accession No. KC182514, which we have named *Ca.* Syngnamydia venezia, see below) in the pCR2.1-TOPO cloning vector or with the empty vector were spotted out onto glass slides (coated with 0.01% poly-L-lysine) and used as positive or negative controls to establish the appropriate hybridization conditions, by increasing the formamide concentrations in 5–10% steps and decreasing the salt concentrations accordingly [38]. This was achieved using a Cy5-labelled chlamydiales-specific Chls-523 probe (S-O-Chls-0523-a-A-18:5′ –CCTCCGTATTACCGCAGC- 3′, used with competitor (com Chls-0523) 5′-CCTCCGTATTACCGCGGC-3′, as previously described by Poppert and colleagues [38] as well as a 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) 5′-labelled eubacterial oligoprobe (Eub338:5′ –GCTGCCTCCCGTAGGAGT- 3′, binding site in *E. coli* 16S rRNA gene 338 to 355) [39] to use as a positive control on *E. coli* and the isolated inclusions. Based on these conditions, cysts, isolated in the same manner as for the sequencing, were incubated at 46°C with FLUOS-Eub338 and Cy5-Chls-0523, in hybridization buffer containing 40% formamide. Detection was by means of a confocal laser scanning microscope (CLSM, Leica TCS SP5, Leica Microsystems). FLUOS and Cy5 were sequentially excited with the 488 nm and 631 nm laser lines respectively, with emission signals collected between 495–570 nm and 640–700 nm. 3D image stacks were collected sequentially (to prevent green–red channel cross-talk) according to Nyquist criteria (voxel size, x:y:z = 277 nm:277 nm:294 nm), processed by deconvolution using HuygensPro via the Huygens Remote Manager v2.1.2 (SVI, Netherlands) and prepared for publication using Imaris 7.6.1 (Bitplane, Zurich, Switzerland).

Results

Broad-nosed pipefish (*S. typhle*) gills were prepared free from surrounding tissues using aseptic conditions under the dissecting microscope (Fig. 1). The epitheliocystis lesions could be readily seen as large (40–300 µm) oval, pale pearlescent-coloured cysts anchored to the gill lamellae. Histological examination of gill tissue revealed multiple intracellular cysts in enlarged epithelial cells. These cysts were not associated with any obvious changes in the gill epithelium or any inflammatory reaction. The cysts appeared to be the result of displacement of the cytoplasm and nucleus by a large (40–300 µm diameter), well-marginated vacuole containing basophilic granular material (Fig. 1b). There were also multiple 20–30 µm hat-like parasites between the lamellae, each with a basal rim of cilia and a central basophilic elongated nucleus (*Trichodina* sp.) (Fig. 1c).

Using pairs of sterile injection needles, individual cysts were dissected free of associated lamellar tissue for analysis (Fig. 2). Puncturing an isolated cyst with a needle (Fig. 2a-c) released large numbers of fine granular particles (Fig 2c), consistent with bacteria of approximately 1–2 µm in size. To establish whether the bacteria within the cysts were members of the order *Chlamydiales*, we performed fluorescence *in situ* hybridisation (FISH) on isolated cysts using a general eubacterial oligoprobe, Eub338, labelled with carboxyfluorescein (FLUOS) [39] (Fig. 2d,f) and with an order

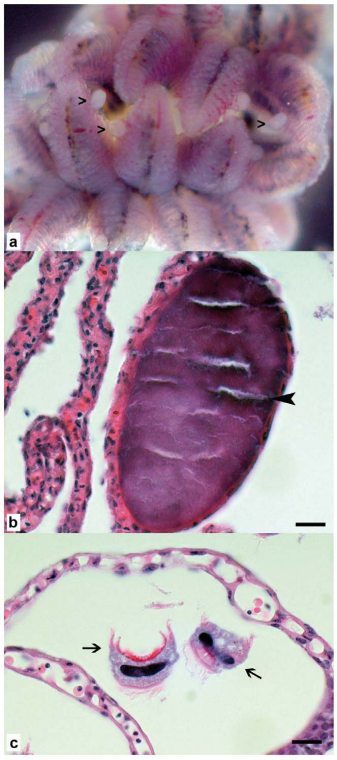

Figure 1. Wet mount and histology of pipefish gills. a: Wet mount in sterile sea water with numerous protruding epitheliocystis lesions clearly visible (open arrowheads). b: Gill lamella with focal intracellular cyst in epithelial cell, 40 µm long axis, with dark basophilic granular material (black arrowhead), the epithelium of affected lamella shows no pathological changes and no inflammatory reaction, scale bar = 10 µm. c: multiple *Trichodina* sp. between lamellae (arrows), no associated pathological changes, scale bar = 10 µm.
doi:10.1371/journal.pone.0070853.g001

Chlamydiales-specific oligoprobe, Chls-523, labelled with Cy5 [39] (Fig. 2e,f). As there was a limited supply of isolated cysts, conditions for hybridisation were established using *E. coli* TOP10 transformed with the full-length 16S rRNA gene 1500 bp fragments of *Waddlia chondrophila* [4] or of the major consensus amplicon (GenBank Accession No. KC182514, which we have named *Ca.* Syngnamydia venezia, see below) in the pCR2.1-TOPO cloning vector or with the empty vector. Both probes labelled the bacteria within the intact cysts, with the better labelling obtained with the *Chlamydiales*-specific Chls-523 probe, indicating that a sequence analysis of the isolated cysts should reflect this dominance.

Whole gill branches containing cysts and individual isolated cysts were prepared for molecular analysis from three individual fish collected in September 2011. Full-length chlamydial 16S rRNA gene PCR amplification of a whole gill branch containing multiple epitheliocysts from each fish as well as amplificates from individual isolated inclusions generated bands of ca. 1500 bp. These products were cloned using TOPO-TA and individual

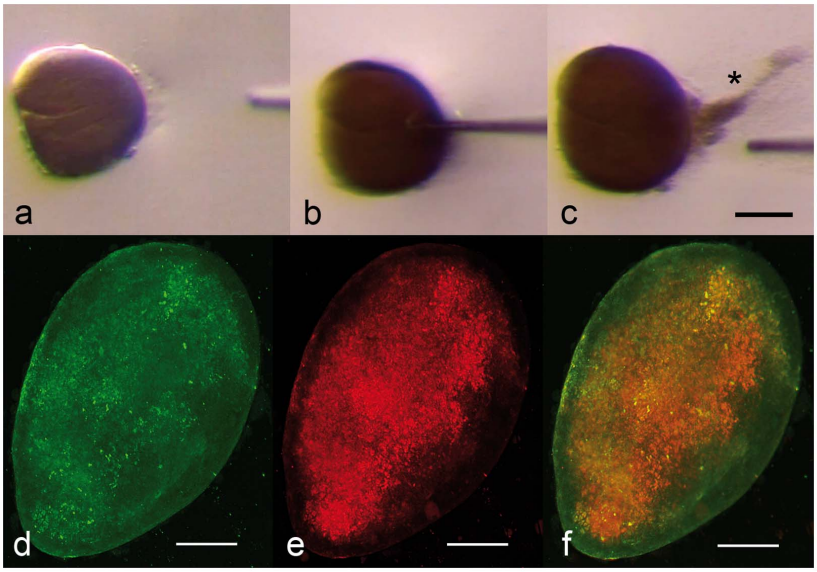

Figure 2. Wet mount and FISH of isolated inclusions. a-c: Wet mount of freshly isolated cyst in sterile sea water (a), punctured with a glass microinjection needle (b), releasing a thick cloud of bacteria (* c). FISH of isolated cyst, labelled with a eubacterial probe (green, d) and a *Chlamydiales*-specific probe (red, e), with the combined signals (f). The FISH images were collected as 3D image stacks by CLSM, deconvolved and the resulting image was compressed into a single 2D-image, shown here. Scale bars = 50 µm.
doi:10.1371/journal.pone.0070853.g002

clones were purified and sequenced. A single full-length consensus sequence was obtained for a total of eleven clones. To test for overall uniformity, we applied the same screening procedure using the shorter 280 bp chlamydial signature sequence. Of a total of 117 individual clones sequenced, all but eight could be assigned to the same single consensus sequence identified in the eleven clones of the full length 16S rRNA gene. Of these eight, three clones were 100% identical to *Ca.* Clavochlamydia salmonicola. The remaining five sequences had 97–98% nucleotide identity to various uncultured *Chlamydiales*, listed in NCBI as unpublished and originating from diverse marine hosts including a sponge (*Callyspongia diffusa*), a mollusc (*Mya arenaria*) and Atlantic salmon (*Salmo salar*).

The major consensus clade comprising 109 sequences, is novel and the corresponding full-length 1477 bp fragment is most closely related to a *Chlamydiales* symbiont of *Xenoturbella westbladi* [40] (96%, 1418/1479 bp), *Candidatus* Fritschea eriococci (94%, 1401/1484 bp) and *Candidatus* Fritschea bemisiae (94%, 1400/1487 bp) [41] and *Simkania negevensis* (93%, 1390/1492 bp) [10] (Fig. 3). Curiously, part of the sequence has 96% (1288/1345 bp) identity to an unpublished Chlamydiales symbiont of *Salmo salar* isolate D261006. According the criteria of Everett and colleagues [42], a 90% or higher 16S rRNA gene sequence homology places a chlamydial sequence within an extant family, with a cutoff of 95% for a new genus. On this basis, the novel *S. typhle* chlamydial endosymbiont likely represents a new genus, within the family *Simkaniaceae*.

To establish whether the novel chlamydial pathogen shares ultrastructural similarities with other members of the *Simkaniaceae*, we prepared TEM images of individual inclusions. All inclusions examined revealed a uniform population of particles (Fig. 4). Individual bacteria averaged 0.76±0.08 µm (SD, n = 42) in width and 2.17±0.44 µm (SD, n = 42) in length, when measured over several inclusions, although the longest bacterial bodies appeared to be dividing cells. In high-resolution images, individual bacteria exhibit an outer electron dense layer bounded by a rippled outer membrane. The bacterial cytoplasm was granular, often containing one or two clusters of electron-lucent regions, which did not appear to be delineated by a membrane. In contrast to the typical uniformly rounded forms of free-living gram-negative bacteria, the endosymbionts often exhibited tight angular forms, possibly indicative of a flexible outer membrane responding to the shapes or pressures exerted by the tight packing of adjacent bacteria in the inclusion. The epithelial cell inclusion membrane was moderately convoluted and 0.5–0.7 µm thick, delineated on the outside by a fine membrane, but with no clear inner membrane evident. We could find no evidence for spikes or bridges connecting the inclusion membrane to the endosymbionts, as we previously described for *Candidatus* Clavochlamydia salmonicola [24]. Bacterial particles appeared to be closely associated with, or even embedded within, the inclusion membrane. Pairs of electron-lucent clusters within endosymbionts, possibly indicative of dividing forms, could be found within bacteria throughout the inclusion. Similarly, longer (>2 µm) forms could be found well separated from, as well as adjacent to, the inclusion membrane.

Discussion

The origins of the phylum *Chlamydiae* lie deep in the evolution of eukaryotes. Our knowledge of chlamydial biology stems almost entirely from a single family, the *Chlamydiaceae*, which are pathogens of land vertebrates, and little is known about how these pathogens have evolved from pathogens of fish, the only

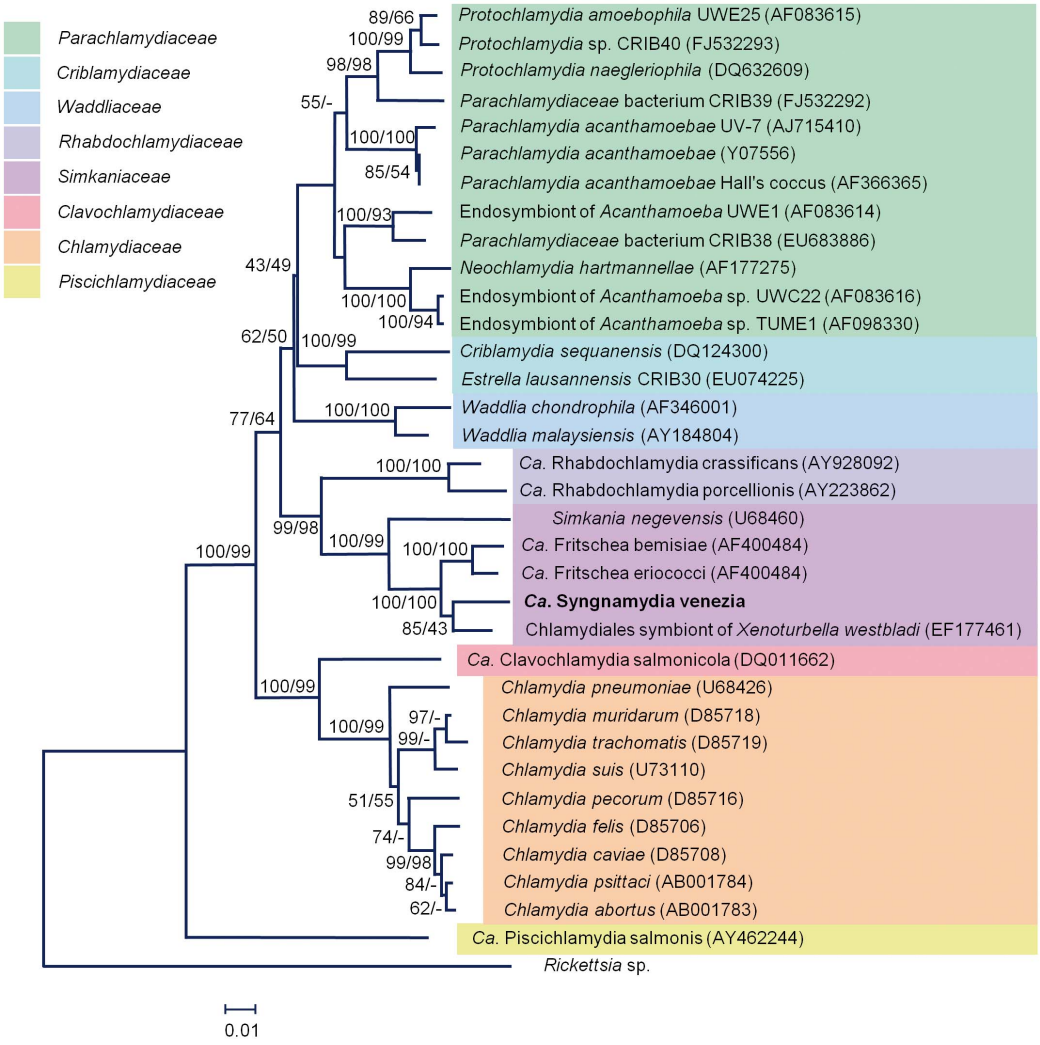

Figure 3. Phylogenetic tree of members of the phylum *Chlamydiae*, including epitheliocystis agents, calculated using NJ and MP analysis of the full length 16S rRNA gene sequence (1477 bp), using *Ricksettia* sp. as an outgroup. The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test (1000 replicates) is indicated. Edited sequences were aligned in ClustalW2. Bootstrap values of 85%, and 43% in the NJ and MP trees, respectively, separated the new species from its closest relatives in the *Simkaniaceae* family.
doi:10.1371/journal.pone.0070853.g003

vertebrate hosts for the *Chlamydiae* from the Ordovician through until the Permian (488 - 299 million years ago). Epitheliocystis, the disease caused by members of the *Chlamydiae* in modern-day fish, has been described for well over 50 different fish species [17,18,19], although there are only a few cases linking morphological and molecular evidence. It has been suggested that the earliest *Chlamydiae* evolved to utilise the niche provided by single cell eukaryotes, in particular free living amoebae [7,15], subsequently using the new skill set acquired to expand into multicellular animals. If this were generally the case, we would expect that a phylogenetic analysis of chlamydial endosymbionts of amoeba would reveal these to be the most ancient. Curiously, the most deeply branching member of the phylum *Chlamydiae* (Fig. 3) is an endosymbiont of fish, *Ca.* Piscichlamydia salmonis [15,20]. In our own studies [19,24], we could find no morphological evidence so far for a biphasic life-cycle of *Ca.* Piscichlamydia salmonis, raising the question as to whether this may be an evolved trait. To answer this, we need to improve our understanding of the biology of fish chlamydial endosymbionts. The results reported here are one step in this direction.

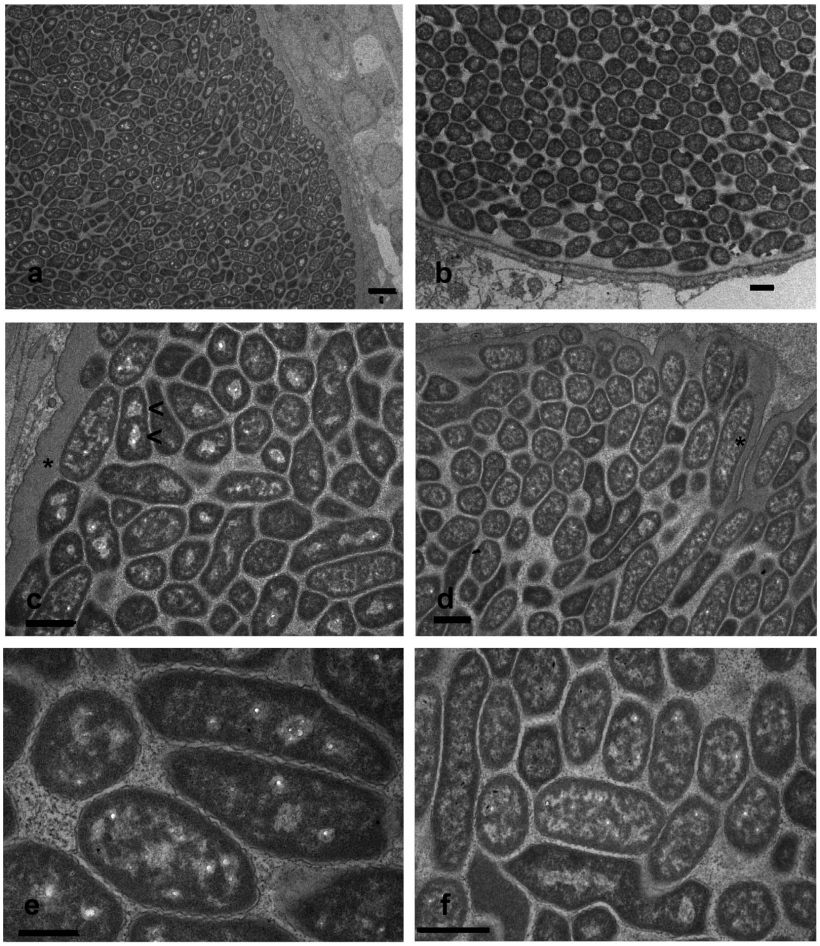

Figure 4. Transmission electron microscopy of epitheliocystis lesions showing features typical for *Candidatus* Syngnamydia venezia. Both a and b give an overview of the dense cell packing. In c and d, endosymbionts showing one or two electron-lucent regions (<), possibly representative of dividing bacteria, and maximally 3.4 μm (d, *) in length. The rippled outer membrane (e) as well as the angular forms (f) are typical characteristics. Scale bars = 1 μm.
doi:10.1371/journal.pone.0070853.g004

All previous efforts to isolate chlamydial endosymbionts from epitheliocystis lesions have proven unsuccessful. We have observed that members of the *Syngnathidae* (pipefish and sea horses) are susceptible to epitheliocystis, own observations and [21], including *S. typhle* collected from the Mediterranean sea, near Venice (this manuscript) and from the west coast of Sweden, near Göteborg [19,30]. Due to the relatively large size of the epitheliocystis inclusions of *S. typhle* and their attachment to what appears to be a single epithelial cell of the fine secondary gill lamellae, without tissue thickening or hyperplasia, it was possible to dissect them cleanly from the gills under aseptic conditions for detailed characterization, allowing us to identify the aetiological agent by PCR-amplification and cloning of the full length 16S rRNA gene from isolated inclusions from three different fish using *Chlamydiae*-specific primers. The eleven clones sequenced differed at only a single site over their 1477 bp 16S rRNA gene sequence, indicating a single species. This result was confirmed by PCR amplification and cloning of the shorter 16S rRNA gene (~280 bp) signal sequence from these samples, where 109 of 117 clones could be assigned to the same consensus sequence. Phylogenetic analysis places this species within the family *Simkaniaceae*, with 93–96% nucleotide identity to other members of this group, indicative of a new species [42]. The phylogenetic tree based on full-length 16S rRNA gene sequences agrees well with recent efforts to reconstruct relationships within the phylum *Chlamydiae* [8,14,20,22,23,41,43,44]. A more thorough phylogenetic analysis would require analysis of additional genes, something we intend to pursue using next-generation sequencing technologies directly

from individual inclusions, an analysis which is beyond the scope of the present report.

Pleomorphic Morphology and Lack of Elementary Bodies

Given the phylogenetic position of the new sequence within the *Simkaniacea*, it was of interest to examine the ultrastructural morphology of this new chlamydial endosymbiont for comparison with published data for other members of the *Simkaniaceae*. Most striking is the pleomorphic, elongated form of approximately 0.75 μm width and 2 μm length, bounded by a rippled outer membrane and a granular electron-dense region extending into the bacterial cytoplasm, where it is interspersed with electron-lucent regions. Similar particles are found closely embedded within the inclusion membrane as well as dispersed throughout the inclusions. These cysts approximate flattened triaxial ellipsoids in form, so that a 50 μm×30 μm×20 μm inclusion would have an estimated volume of 15700 μm^3 (volume = $4/3\pi abc$, where a, b and c are the lengths of the half axis) and could contain 4,000–8,000 bacteria, depending on packing density.

We found no evidence of smaller electron-dense forms typical for EBs as have been described for other members of the *Simkaniaceae*. *Simkania negevensis*, originally isolated from cell cultures and subsequently shown to be a human respiratory pathogen especially common within the Negev region of Israel [45,46,47], was reported to exhibit both smaller electron-dense forms and larger replicative bodies, the latter similar in shape to the pleomorphic forms described here [46]. From the limited evidence available, a single figure from the original publication reproduced in Everett et al. [41], *Ca.* Fritschea bemisiae, an endosymbiont of insect gut epithelium, may also have similar replicating bodies to *S. negevensis* and possibly also condensed elementary bodies. A range of forms have been described for a *Chlamydiales* endosymbiont of phagocytic gastrodermal cells in the marine deuterostomes, *Xenoturbella westbladi* and *Xenoturbella bocki* [40], postulated to represent three different reticular bodies and two infectious or elementary bodies. The reticular bodies had in part a similar rippled outer membrane and a flexible outer membrane whose shape appears to adapt to the packing pressure of the surrounding bacteria.

Conclusions

The *Simkaniaceae*, a single family within the phylum *Chlamydiae*, is pathogenic to hosts ranging from a primitive eukaryote described as being merely a "ciliated bag with epithelial epidermis and gastrodermis and a mouth" [40], through to insects, humans and now fish. To what degree the different morphologies of the *Simkaniaceae* are adaptations to markedly different cellular niches in such phylogenetically divergent hosts must await further studies, which will require their successful isolation for cultivation. The method described here for preparation of the inclusions, free from substantial remnants of host tissue, may aid in these efforts. Particularly opportune is the advent and annotation of the *S. negevensis* genome sequence [8]. This resource may well provide the scaffold for comparative genomics of members of the *Simkaniaceae* using direct sequencing technologies, enhancing our understanding of chlamydial evolution. Given this wide host range, the possibility of zoonotic transfer of members of the *Simkaniaceae* is not to be underestimated, and something which could increase the utility of this clade as a model for the study of host-pathogen evolution. Pipefish of the *Syngnathidae* feed largely on small crustaceans and plankton [48,49] and are especially prevalent in the seagrass beds known to provide a critical habitat for many commercial fisheries and support a high diversity of invertebrate and fish life [50,51]. These same areas also provide an important buffer between catchment areas from human settlements, land-based agriculture and near shore aquaculture and more pristine offshore marine or reef environments. Monitoring pathogen loads of important indicator species may be of use in efforts to sustainably manage these threatened biotopes and aid the parallel development of adjoining, sustainable aquaculture [52]. Future work will aim to further characterise this novel chlamydial endosymbiont, *Ca.* Syngnamydia venezia, and to take advantage of the existing knowledge on the genetic structure of European populations of *Syngnathus typhle* [30], to explore the genetic affinities between this putative pathogen and its host.

Description of "Candidatus Syngnamydia venezia"

"*Candidatus* Syngnamydia venezia" [Syng.na.my'di.a L. F. n. *Syngnathus* name of host genus and *Chlamydiae* name of bacterial phylum; L. F. n. *Syngnamydia*, *Chlamydiae* originating from Pipefish; ve.ne.zi.a L. M. n. *Venezia,* lagoon from which the samples were collected].

The provisional taxon "*Candidatus* Syngnamydia venezia" contains intracellular bacteria that may infect gill cells of *Syngnathidae* in marine environments. Members of the taxon exhibit morphologies resembling other members of the *Simkaniaceae*, but as appears to be the case for at least one other *Chlamydiae* infecting fish hosts [19,24], may not have a biphasic life cycle. The pleomorphic RBs are approximately 0.75 μm in width and 2 μm long, with longer forms of up to 3.5 μm likely replicating bodies shortly before division. In each RB, a rippled outer membrane bounds an electron-dense membrane-proximal layer surrounding a granular cytoplasm interspersed with electron-lucent regions, which can coalesce to form one or two clusters, the latter possibly indicative of dividing cells. The outer membrane appears to be quite malleable, the shape ranging from round to angular rods and appearing to fill the space provided by the stacking of the surrounding RBs. No clear EB or infectious particle with an electron-dense core was observed, raising the possibility that the RBs may themselves be able to initiate an infection, and lead to epitheliocystis in syngnathid hosts. The gill epithelial inclusions do not appear to cause tissue inflammation or hyperplasia and approximate flattened triaxial ellipsoids in form with a major axis ranging from 40–100 μm. The 16S rRNA gene of "*Candidatus* Syngnamydia venezia" has been deposited in GenBank (Accession No. KC182514). The 16S rRNA gene shows phylogenetic affinity towards the family *Simkaniaceae*.

Acknowledgments

The authors wish to thank members of the Wilson lab, in particular Jan Knott and Alexandra Wegmann for many helpful discussions and for fish capture and care.

Author Contributions

Conceived and designed the experiments: AF EW HSP LN AW MS DR HS AP LV. Performed the experiments: AF EW HSP LN MS DR LV. Analyzed the data: AF EW HSP LN AW MS DR HS AP LV. Contributed reagents/materials/analysis tools: AF EW HSP LN AW HS AP LV. Wrote the paper: AF EW HSP AW HS AP LV.

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Chapter 3

Emerging epitheliocystis in farmed gilthead seabream: identification, characterisation and genomes of novel beta-proteobacterial agents

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3.1 Abstract

Aquaculture provides a large and increasing source of food protein globally, at risk from emerging disease. The gill infection epitheliocystis is causing increasing mortalities in gilthead seabream, a heavily farmed fish species in the Mediterranean. Epitheliocystis is generally associated with chlamydial bacteria, but these current infections are caused by two previously unidentified beta-proteobacterial species. We have characterised these coinfecting intracellular bacteria using high resolution imaging and genomics, presenting the most comprehensive study on epitheliocystis agents to date, even in the absence of cultured bacteria. The genomes of the two species, *Ca*. Ichthyocystis hellenicum and *Ca*. Ichthyocystis sparus, have been de novo sequenced and annotated, and show a compact core genome with an unprecedented number of tandemly arrayed gene families. This study represents a critical insight into novel, emerging fish pathogens which can be used to underpin future investigations as well as diagnostic and treatment strategies.

3.2 Introduction

Aquaculture is an expanding industry, growing to meet the consumer demand for high quality food protein, producing 66 million tonnes globally (2012; (F.A.O., 2014)). Gilthead seabream (*Sparus aurata*) is a high-value fish species, with over 130,000 tonnes farmed globally in 2010 (F.A.O., 2012), and is the dominant species within Mediterranean aquaculture. As intensity of farming and stocking densities increases, aquaculture farms are at ever greater risk from infectious diseases (Segner et al., 2011). Some of the most common diseases to affect gilthead seabream result from bacterial (Vibriosis, Photobacteriosis), viral (Lymphocystis) and parasitic (Monogeneans) infections (Colorni and Padros, 2011), but epitheliocystis is now evident as an emerging disease, with increasingly severe episodes seen each year.

Epitheliocystis was first described in 1969 as cysts in the gill epithelia of the bluegill (Hoffman et al., 1969) and has since been identified within many fish species: wild and farmed; freshwater and marine (Nowak and LaPatra, 2006; Stride et al., 2014). The most common causative agents of epitheliocystis have been identified as species within the phylum Chlamydiae (Draghi et al., 2007; Draghi et al., 2004; Fehr et al., 2013; Karlsen et al., 2008; Schmidt-Posthaus et al., 2012; Steigen et al., 2013; Stride et al., 2013a; Stride et al., 2013b), including those more closely related to the human infecting and zoonotic *Chlamydia* species (Karlsen et al., 2008) and those found in a more divergent clade within the phylum (Draghi et al., 2004; Stride et al., 2013a; Stride et al., 2013b). In recent cases, two non-chlamydial bacterial agents have been implicated (Mendoza et al., 2013; Toenshoff et al., 2012). However, no agents of epitheliocystis have yet been cultured and all bacterial identification to date has relied solely upon sequencing of 16S rRNA genes and *in situ* hybridisation of derived probes to infected tissue sections.

The disease has been investigated in the past within both wild and cultured gilthead seabream from the Red Sea coast of Israel (Paperna, 1977; Paperna et al., 1981) and Spain (Crespo et al., 1999), including detailed gill pathology with electron microscopy, but no molecular work was performed in these studies and no causative agents were identified. Although *Chlamydia*-like agents were implicated in these studies, as analysis of electron micrographs of cysts appeared to give evidence of a chlamydial lifecycle, immunohistochemistry of infected *Sparus* gills with anti-chlamydial LPS antibodies produced no reaction (Crespo et al., 1999).

In farmed gilthead seabream in the Mediterranean, epitheliocystis appears to be a seasonal disease occurring during the warmer months and often breaks out when juvenile fish are first introduced to sea cages. An increasing trend of epitheliocystis has been seen over the past few years [Dr Kantham Papanna, Branch Officer (Greece) of the European Association of Fish

Pathologists, personal communication] with the disease also increasing susceptibility to secondary infections [Dr Panos Varvarigos, VetCare, Athens, Greece, personal communication].

We have studied this disease in several fish farms around Greece, one of the largest aquaculture producing countries of the Mediterranean . By dismissing previous presuppositions and using rigorous methods, we have identified a novel beta-proteobacterial genus, implicated as a major cause of epitheliocystis in gilthead seabream. This uncultured emerging pathogen has been characterised molecularly and microscopically from infected gill tissue, with draft genomes providing insights into the diversity and lifestyle of these new bacteria. The approach we describe, of direct analysis of lesions, would also prove valuable for obtaining detailed morphological and genomic data from other novel uncultured infectious or symbiotic microorganisms.

3.3 Materials and Methods

3.3.1 Sample collection

All gilthead seabream gill samples were provided by Dr Nancy Dourala, expert veterinarian and fish pathologist from Selonda. This is a pioneering Greek aquaculture company, founded in 1981, currently producing over 20,000 tonnes gilthead seabream per year from over 100 million juveniles, in addition to sea bass. Samples from November 2012 (Argolida, Saronikos) were taken during a low level infection, whereas samples from 2013 (Argolida, June, from 4 cages, numbered 11-45 reflecting cage number in the first digit, and Arkadia, October, 2 cages sampled without prior checking for epitheliocystis) were selected during epitheliocystis outbreaks. Infection levels are determined by the pathologist based on the number of cysts per gill arch, number of gill arches infected, number of fish infected per cage and mortality attributed to the disease. Features of the fish from the sampled cages are given in Table 1. Gill arches from individual morbid fish were taken in parallel into 10% buffered formalin, RNALater and pure ethanol, with the 2013 samples also taken into M4RT Chlamydia transport medium (Remel Microtest M4RT, Thermo Fisher) and sterile sea water (SSW). Samples were transported cooled overnight for laboratory processing.

Mortality is estimated through the pathology of dead fish retrieved every few days from the bottom of the cages.

3.3.2 Sample processing

Microscopy: Formalin fixed samples were embedded in paraffin (FFPE) after dehydration in ascending alcohol concentrations ending in xylol solution, with 3 mm sections mounted on

positively charged glass slides used for routine haematoxylin and eosin staining (HE), Periodic acid–Schiff (PAS) staining, and fluorescent *in situ* hybridisation (FISH, described below).

Culture: On arrival in Zurich, chilled samples collected into M4RT or SSW were microdissected into infected gill filaments or single cysts using a Leica M165C dissecting microscope. Cysts from SSW were washed briefly in cold PBS with 50 mg/ml vancomycin and 1 mg/ml amphotericin B. Whole or homogenised cysts were placed into prepared cultures of fish epithelial cell line EPC (Winton et al., 2010) monolayers in minimal essential medium (MEM) containing 10% fetal calf serum (Gibco, Life Technologies) or *Acanthamoeba castellanii* ATCC 30010 in peptone yeast extract glucose (PYG) broth (Greub et al., 2004) in 24 well trays and incubated for up to one month at 28 or 32 ^{o}C respectively. The cultures were checked regularly for putative intracellular bacterial growth by phase contrast microscope.

DNA extraction: DNA was extracted from gill fragments or filaments stored in RNALater or ethanol using the Qiagen DNeasy Blood and Tissue kit, eluting into 100ul HPLC water.

3.3.3 Bacterial identification

Identification of bacteria present was carried out using either Chlamydiae-specific 16S rRNA gene primers (Everett, 2005) (Draghi et al., 2004) or universal bacterial primers (Weisberg et al., 1991). Positive bands were analysed further only when template free negative controls showed no signal. 16S rRNA gene amplicons were sequenced from the PCR product or cloned into Topo vector pCR2.1 prior to capillary sequencing from both ends (Microsynth, Balgach, Switzerland). The resulting reads were assembled (CLC Main Workbench 7.0.2 (CLC bio, Qiagen) and Sequencher 5.2.4 (Gene Codes Corporation, USA)), compared using blastn against the Genbank database and used to create alignments with reference sequences (using Muscle and PhyML v3 within Seaview v4 (Gouy et al., 2010)). Representative 16S rRNA gene sequences have been deposited with EMBL with the following accession numbers: LN612726-LN612730 representing 2013Arg42i, 2013Ark19i, 2013Arg22i, 2013Arg32i, 2012Sar4i, respectively (the i subscript indicates *Ca*. Ichthyocystis sequences).

3.3.4 Quantitative PCR

The chlamydial 16S rRNA gene sequences obtained were used to modify the Chlamydiae-specific Taqman qPCR protocol (Lienard et al., 2011) (forward: 5'-CCGCCAACACTGGGACT-3', reverse: 5'-GGAGTTAGCCGGTGCTTCTTTAC-3', probe: 5'-FAM-CTACGGGAGGCTGCAGTCGAGAATC-BHQ-3'). A qPCR protocol was also designed against the 16S rRNA gene from the *Ca.* Ichthyocystis genus (forward: 5'- CAAGGCGACGATCGGTAGCTG-3', reverse: 5'-

TTACAACCCTAAGGCCTTCTTCACC-3', probe: 5'-FAM-TTGCTGGATCAGGCTTCCGCCCATTGTCCAAA-BHQ-3'), allowing quantification of infected material, but with decreased sensitivity at lower sample concentrations. All Taqman qPCR protocols were designed and validated against serial dilutions of amplicons from target and more distantly related species. Reactions were carried out on a StepOne Plus real-time PCR system (Applied Biosystems). Taqman Fast Advanced reagents (Applied Biosystems) were used according to the manufacturer's instructions with 1-5 ml input DNA in a total reaction volume of 20 ml.

3.3.5 Fluorescent staining and imaging

Fluorescent in situ hybridisation (FISH) probes used are given in Table 3. FISH was performed using the Ventana Discovery XT automated platform for consistency of results. Automated deparaffinisation was performed and pretreatment with 0.2M HCl in PBS followed by pepsin (500 mg/ml in this buffer) for 4 min. Following addition of 50 ng probe per slide in a hybridisation buffer comprising 6x SSC, 5x Denhardt's solution and 12% dextran sulphate, samples were denatured at 90^{o}C for 4 min and hybridised at 48^{o}C overnight. A wash with 2xSSC at 48^{o}C was followed by manual post-staining with 4'-6-diamin-2-phenylindole (DAPI) at 10 mg/ml to visualise bacterial and host DNA, and concanavalin A-AlexaFluor488 or -AlexaFluor546 (25 mg/ml) (Life Technologies) for 10-30 minutes. Negative controls for probes included sections of fish tissue not infected with *Ca*. Ichthyocystis, as determined by PCR and qPCR. Immunofluorescent (IF) staining was performed on sections following standard antigen retrieval in citrate buffer using anti-sodium-potassium-ATPase antiporter a5 (Developmental Studies Hybridoma Bank, University of Iowa) with AlexaFluor546-labelled secondary antibody (Life Technologies), or on rehydrated sections permeabilised with 0.1% Triton X-100 in PBS using anti-OxPhos complex IV subunit I mouse antibody (Life Technologies) diluted in 2% BSA and AlexaFluor488-labelled secondary antibody (Life Technologies). IF slides were also counterstained with DAPI as above. FISH, IF and HE sections were scanned on a Hamamatsu Nanozoomer 2.0HT scanner for an overview of the section, and high resolution imaging was performed on a confocal laser scanning microscope (SP5, Leica Microsystems). Deconvolution was performed using Huygens (Scientific Volume Imaging, Netherlands) (Ponti et al., 2007), image preparation was performed with Imaris 7.6.1 (Bitplane, Oxford Instruments) and Photoshop CS4 extended, Version 11.0.2 (Adobe).

Probe	Specificity (sp)	Sequence / fluorophore	Position (*E. coli* numbering)	Reference
Chls-0523	Phylum Chlamydiae	5'-CCTCCGTATTACCGCAGC-3'Atto488	524-541	(Poppert et al., 2002)
Pisci-0312	*Ca.* Piscichlamydia/ Similichlamydia	5'-AGTCCCAGTGTTGGCGATCG -3'Cy3	304-323	This study
Ichthyo-290	*Ca*. Ichthyocystis genus	5'-CATCCTCTCAGACCAGCTACCGATC-3'Cy3	281-305	This study
Ichthyo-230	*Ca*. I. hellenicum	5'-GGTCATCGGCCGCTCCTATCGC-3'Cy3	220-241	This study
E-474	Control: gamma-proteobacteria	5'- AACCTTCAACCTTTCCTCCC-3'Cy3	445-464	This study

Table 3 *Probes used for FISH based on target bacterial 16S rRNA gene sequences.*

3.3.6 Electron Microscopy

Scanning electron microscopy (SEM): Samples were washed with sodium cacodylate buffer, post-fixed with OsO_4 and dehydrated in an ascending alcohol series, mounted on stubs, and sputter coated with gold-palladium. They were viewed using a JEOL JSM-6390LV scanning electron microscope at 15 kV at the Electron Microscopy Laboratory of the University of Crete, Heraklion.

TEM: Samples were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide (OsO_4), buffered with 0.1M sodium phosphate (pH 7.4). Samples were then dehydrated in an ethanol series, embedded in Epon 812 resin, and ultrathin (90 nm) sections were stained with uranyl acetate and lead citrate. Images were acquired using a Philips CM10.

Focused ion beam-scanning electron microscopy (FIB-SEM): Sample blocks were fixed and prepared as for TEM. Semithin and ultrathin sections were obtained to identify the regions containing cysts. The selected blocks were attached to 12mm stubs by conductive carbon cement followed by carbon coating. 3D datasets were acquired with a FIB-SEM Auriga 40 Crossbeam (Zeiss, Oberkochen, Germany) using the FIBICS Nanopatterning engine (Fibics Inc, Ottawa, Canada). The gallium-ion beam for milling was used at 30kV, 600pA current and the images were acquired at an acceleration voltage of 1.5 kV using an in-lens energy selective backscattered electron detector (ESB) with a grid voltage of 1.3 kV. The resolution was set to 5 nm in the XY axes

and 5-10 nm in the Z axis. The image stacks were aligned with TrackEM2 (Cardona et al., 2012). The aligned dataset was visualized with Imaris 7.6.1.

3.3.7 Genomics

Cysts from fresh or preserved (RNALater, ethanol, M4RT) gill samples with high numbers of cysts were micromanipulated to be as free as possible of contaminating host material using a Leica M165C dissecting microscope. Pooled cysts from individual gill samples were then extracted using the Qiagen DNeasy Blood and Tissue kit. Some samples were subject to host DNA depletion using the NEBNext® Microbiome DNA Enrichment Kit (NEB, MA, USA). Aliquots of samples (1ul) were subject to Genomiphi V2 WGA (GE Life Sciences) and the amount of target DNA in pre- and post-amplification samples was determined using qPCR.

Samples were sequenced using the Illumina Miseq platform with 250bp paired end (PE) reads following Nextera library creation. One run was performed as part of a collaboration with the Wellcome Trust Sanger Institute, U.K. Raw sequencing PE reads were adaptor and quality trimmed and filtered using Trimmomatic version 0.32 (Bolger et al., 2014). Resulting fastq files were screened by mapping with SMALT [http://www.sanger.ac.uk/resources/software/smalt/] against the 16S rRNA gene sequences of the candidate epitheliocystis pathogens, including *Ca*. Piscichlamydia and *Ca*. Similichlamydia sequences, to determine the presence of specific pathogens and to estimate genome coverage. Mapping was also used to screen the data against the gilthead seabream mitochondrial 16S rRNA gene sequence (EMBL accession number AF247432) and the gilthead seabream 18S rRNA gene sequence (as identified from a preliminary assembly) to confirm sample origin and determine levels of fish DNA contamination.

De novo assembly of quality controlled reads was performed using SPAdes v 3.1.0 (Bankevich et al., 2012). Sample 2013Ark11 (*Ca.* I. hellenicum; amplified) was run as one of 12-plexed samples on a Miseq run, whereas read data for sample 2013Arg41 (*Ca.* I. sparus; not amplified) was pooled from one run 12-plexed and one of 6-plexed. Assemblies were tested using various K-mers in both single-cell mode and multi-cell mode (Nurk et al., 2013) and quality assessment using QUAST (Gurevich et al., 2013) and CGAL (Rahman and Pachter, 2013) (Tables S3 and S4). For our data set, multi-cell mode produced better assemblies than single-cell mode. This resulted in 2743 scaffolds (4.06Mb) for 2013Ark11 and 1794 scaffolds (3.37Mb) for 2013Arg41.

Removal of scaffolds representing DNA from the host or other bacterial species was performed initially using kmer coverage parameters as given by SPAdes. Coverage was plotted against scaffolds and used to define a cut-off above which scaffolds were most likely to belong to the target bacterium (2013Ark11 cut-off 35x; 2013Arg41 cut-off 30x). Additionally, scaffolds below 1Kb were

discarded as being unlikely to provide valuable information to the assembly. This resulted in 98 scaffolds covering 2.16Mb for 2013Ark11 and 198 scaffolds covering 2.53Mb for 2013Arg41. The two assemblies were aligned against each other to determine the scaffolds shared between the genera, representing the core genome of the novel genus, estimated at 1.5Mb.

Automated annotation using Prokka (Seeman, 2014) resulted in the placement of approx. 1600-1800 CDSs per genome. These were manually checked against Prodigal gene prediction (Hyatt et al., 2010) using Artemis (Rutherford et al., 2000) and ACT (Carver et al., 2005), with a six-frame tblastx search used to investigate additional hits. Further discarding of contaminating or uninformative scaffolds was performed when no identifiable bacterial CDSs were present. Annotation was curated manually in Artemis using blastp identities, Pfam, Rfam, SignalP, TMHMM and ncoils. Analysis was focussed on “accessory” regions not shared between the species, with the “core” sections of the genomes shared within the genus used to coordinate the annotation of equivalent genes. Preliminary analysis of the annotation is presented here, with detailed ongoing manual annotation being prepared for further publication. Iterative Hidden Markov Model (HMM) searches of gene family members were done using hmmer-3.1 (Eddy, 2009). For each manually curated gene family, HMMs were built based on protein alignments and then used to scan genome drafts (reporting e-value cutoff 0.0001). After each scan, new HMMs were built and used, until the set of protein matches stabilized. HMMs were used to scan protein databases NCBI nr [http://www.ncbi.nlm.nih.gov/], Uniprot [www.uniprot.org] and the RSCB PDB [http://www.rcsb.org/pdb].Whole genome phylogenies were constructed by mapping sequence data using SMALT against the closest reference assembly, determining SNP locations and running PhyML phylogeny from resulting pseudo-alignment.

Read data for all ten samples sequenced has been submitted to the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena/) under study PRJEB7439 and the accession number ERR361036 for sample 2013Arg42.

3.4 Results

3.4.1 Sampling and pathology of gilthead seabream

Epitheliocystis infections represent an increasing problem in farmed gilthead seabream in the Mediterranean, with a commercial aquaculture firm observing recent year-on-year increases in mortality attributable to epitheliocystis as described by their own veterinarian and fish pathologist (Figure S1). This is predominantly a short lived infection lasting approximately 2 weeks, resulting nevertheless in mortality rates of up to 20% per sea cage. Observations indicate that after

recovery, fish within the same cage do not suffer recurrent infections. Thus epitheliocystis can be regarded as an emerging disease in the Mediterranean.

To determine the severity and aetiological agents associated with epitheliocystis, gilthead seabream were sampled from commercial fish cages at three coastal locations around Greece on three occasions over a year (Table 1). Argolida (sampled November 2012 and June 2013 during an epitheliocystis outbreak) and Arkadia (sampled October 2013 during an outbreak) are both in the Argolic Gulf, and Saronikos (sampled randomly November 2012) is on the other side of the peninsula in the Saronic Gulf.

Gill samples were screened for signs of disease (Figure S2) and separated gill arches from individual fish were placed into a selection of media for preservation of morphology, nucleic acid and microbial viability. Histopathology demonstrated the level of epitheliocystis infection, cyst characteristics and presence of co-infecting pathogens (Table 1).

Cysts were identified in the gills of all fish, with those sampled during outbreaks displaying high numbers of large cysts varying from 20-100 μm in diameter (Figure 1a). Two major cyst types were identified, all located on the tip or on the base of the secondary lamellae (Figure 1b and c). The first type have basophilic and finely granular content (Figure 1b) and are surrounded by a fine 1-2 μm thick eosinophilic membrane (Figure 1d). A layer of epithelial cells often surrounds these cysts, occasionally showing hyperplasia (Figure 1b). Some cysts can be seen to have a rupture in the eosinophilic membrane and are surrounded by macrophages, lymphocytes and occasionally neutrophils (Figure 1e). Mild inflammation is often seen around the cysts, and in some cases secondary lamellae are blunted and fused. The second type of cyst is smaller, up to 40 μm in diameter with more basophilic and coarsely granular contents, and surrounded by a slightly thicker (2-3 μm) membrane (Figure 1c). Some co-infections were observed in rare cases (Table 1) but these were not thought to contribute greatly to the disease status of the fish. The many large cysts would be likely to affect fish respiration due to their number and the hyperplasia of the epithelium, causing disruption of oxygen diffusion and distress to the host.

Location	Date	# fish sampled	Fish size (mean of each cage)	Cage fish mortality	Infection / cyst description	Co-infection
Argolida	Nov 2012	2	ND	0%	Low infection	
Saronikos	Nov 2012	5	ND	0%	Low-medium infection	Ciliate *Trichodina* Amoebae
Argolida	Jun 2013	20 from 4 sea cages	15-23g	1.3-15.5%	High infection	*Trichodina* Lymphocystis Fungi Flukes
Arkadia	Oct 2013	20 from 2 sea cages	9-11g	2.9-3.1%	Medium-high infection	Flukes

Table 1 *Sampling of gilthead seabream and gill pathology. ND Not determined*

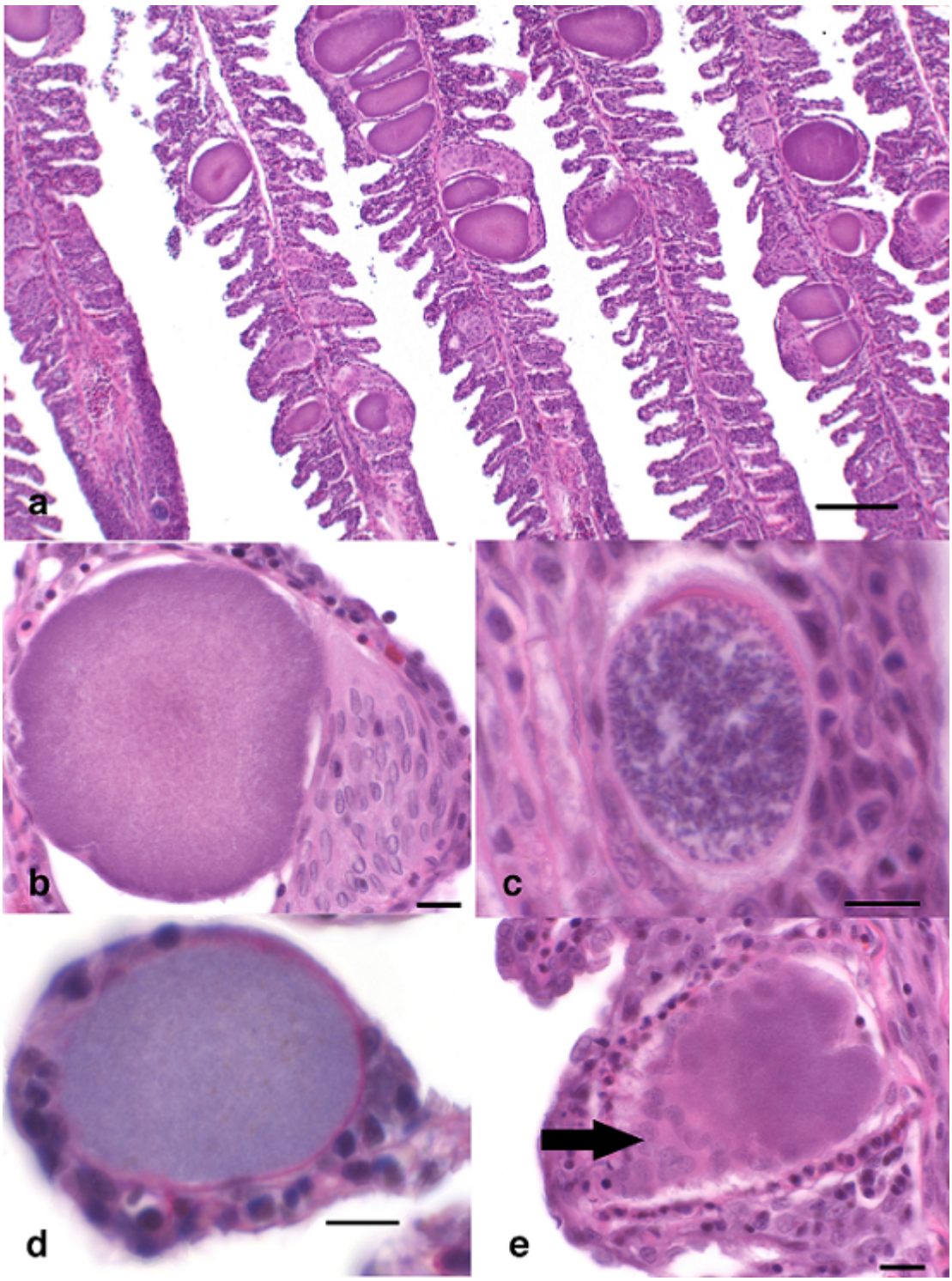

Figure 1 *Sections of representative gills stained for histopathology. a. Overview of gill from fish 2013Arg11 with hematoxylin and eosin (HE) staining. The gills are heavily infected with cysts, up to 100 mm in diameter. Scale bar represents 100 mm. b. Cyst from fish 2013Arg11 with HE staining, located between two fused secondary lamellae. The cells towards the tip of the lamellae can be seen to be atypically thickened and hyperplastic through epithelial proliferation with squamous metaplasia. The epithelial cells have a large amount of eosinophilic cytoplasm and are tightly*

connected to each other. The gap around the cyst is thought to be a fixation artefact through shrinkage. Scale bar represents 10 mm. c. Cyst from fish 2013Arg11 under HE staining showing coarser granular contents and a thicker eosinophilic membrane. Scale bar represents 10 mm. d. Cyst from fish 2013Ark11 under periodic-acid-Schiff (PAS) staining, showing polysaccharide containing membrane (pink). Within the membranous layer an elongated nucleus with finely stippled chromatin is visible. Scale bar represents 10 mm. e. Cyst from fish 2013Arg11 with HE staining showing a ruptured cyst and associated macrophages (arrow). Scale bar represents 10 mm.

3.4.2 Pathogen identification

As Chlamydiae are the most commonly identified agents of epitheliocystis, Chlamydiae-specific primers were initially used to amplify the 16S rRNA gene (Draghi et al., 2004; Everett et al., 1999) from gill tissue extracts from Saronikos (Nov 2012) and Argolida (Jun 2013). Many novel sequences belonging to the *Piscichlamydiales* clade were identified [Seth-Smith *et al*., *Sparus*-Piscichlamydia paper, in preparation]; however, exhaustive FISH analysis could not localise these gene sequences to within the large cysts identified as the pathological lesions in HE (see Supplementary Information and Figure S3). Therefore amplification of DNA extracted from an isolated cyst (fish 2013Arg42) using universal 16S primers (Weisberg et al., 1991) was performed. Two resulting cloned amplicons were found to contain identical sequences representing the 16S rRNA gene of a novel beta-proteobacterium. Database searches showed that this gene sequence shares a maximum of 89.9% nucleotide identity with any previously deposited sequence (Uncultured *Sterolibacterium* sp. clone OTU-X1-28, accession JQ668544), and 89.2% with any cultured and characterised bacteria (*Sterolibacterium denitrificans* strain Chol-1S, AJ306683 (Tarlera, 2003)), indicating that it represents a diverse new genus of beta-proteobacteria. (New genera are described by 86.5-94.5% identity between 16S rRNA genes (Yarza et al., 2014)). Phylogenetic analysis showed that this novel sequence represents a close relative of the recently described epitheliocystis pathogen from farmed Atlantic salmon (*Salmo salar*), *Candidatus* Branchiomonas cysticola (88% nucleotide identity) (Toenshoff et al., 2012) (Figure 2). Using primers designed to amplify this novel 16S rRNA gene sequence, gill samples from fish at each location and timepoint (Table 1) were screened by PCR and sequencing. All samples were found to carry sequences representing this novel genus, and phylogenetic analysis showed that they fall into two distinct clades, sharing only 95.2% sequence identity, indicating that these are divergent species within one novel genus (species distinction defined at 94.5-98.7% 16S rRNA gene nucleotide identity (Yarza et al., 2014)).

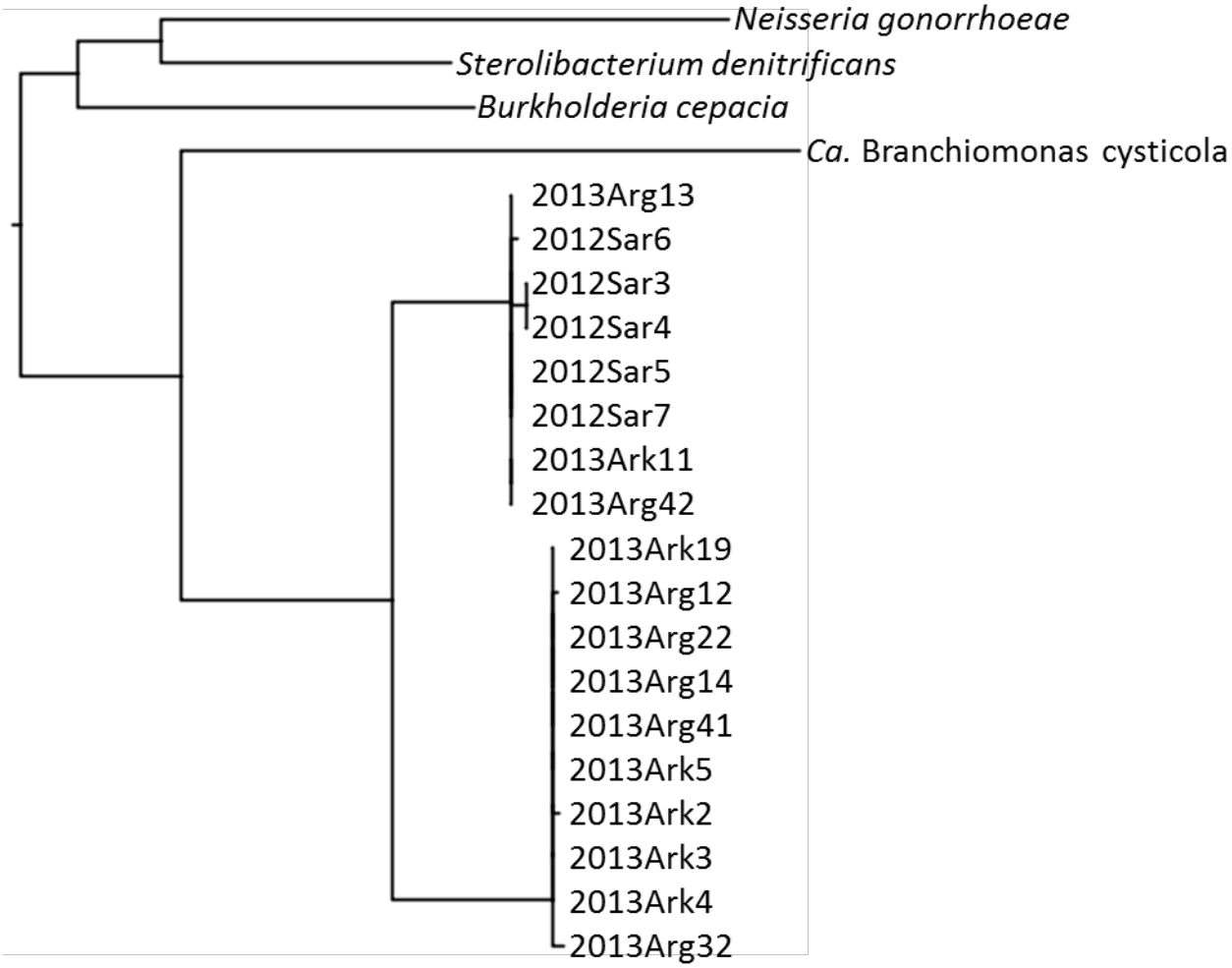

Figure 2 *Phylogenetic analysis of novel 16S rRNA gene sequences from gilthead seabream. Amplified 16S rRNA genes provided up to 1500bp sequence. The alignments and phylogeny were performed within Seaview with 100 bootstraps. Two distinct clades indicate that there is considerable diversity within the novel genus (Ca. Ichthyocystis). Other beta-proteobacteria are included as outgroups, with the epitheliocystis agent Ca. Branchiomonas cysticola (Toenshoff et al., 2012) as the species sharing the most recent common ancestor with the novel bacteria. 2012Arg, 2013Arg, 2013Ark, 2012Sar refer to the year and location of isolation, followed by the fish number.*

We developed a quantitative PCR (qPCR) protocol to enable determination of the load of the new pathogen in gilthead seabream gill samples. When compared with the qPCR values from a Chlamydiae-specific system, low levels of chlamydial sequence were quantified in all samples, but levels of the new pathogen genus were higher in every case, in particular in the heavily infected samples from 2013Arg and 2013Ark (up to 3000x higher, Table S1). However, these bacterial load

values cannot be compared between samples, as gill samples were not standardised prior to DNA extraction. An approximate mean figure of 200,000 genomes per cyst was determined from analysing extracts of multiple microdissected cysts.

Culture onto fish epithelial cell monolayers or *Acanthamoeba* was attempted from samples sent in transport medium or sterile sea water (SSW). However, most of the wells became overgrown by contamination and no intracellular growth was seen in the wells which were not contaminated.

3.4.3 Localisation of bacteria in infected tissue

Fluorescent *in situ* probes were designed to hybridise to the new 16S rRNA sequences (Ichthyo-290) and to those of the gilthead seabream gill-derived 16S rRNA sequences from the *Piscichlamydiales* clade. Comparison of hybridisations (FISH), performed on sections from 18 fish, under standard automated conditions, showed that the dominant cysts identified pathologically produce a very strong signal using the probes against the novel bacterial genus (Figure 3) compared with no signal against *Ca*. Piscichlamydia clade probes (Figure S3) and those targeting more diverse bacteria (data not shown). This indicates that these novel pathogens are responsible for the large cysts throughout the gill filaments, and are the dominant cause of epitheliocystis in these gilthead seabream around the Greek coastline, over at least one year. As uncultured, novel pathogens, the two species were named *Candidatus* Ichthyocystis hellenicum and *Candidatus* Ichthyocystis sparus (see Taxonomy section).

A further *in situ* probe (Ichthyo-230) was designed to discriminate between the two species, hybridising to *Ca.* I. hellenicum sequences and containing 5/22 mismatches to *Ca.* I. sparus sequences such that these will not hybridise under the conditions used. Screening of gill sections from 16 fish with this probe showed that both species can be found in the same gill section indicating that they are co-circulating pathogens (Figure 4). Both species were identified using FISH in the heavily infected Argolida 2013 and Arkadia 2013 samples, whereas only *Ca.* I. hellenicum could be identified in the samples from 2012 (Table S2). Comparison of serial sections stained with HE and FISH show that the cysts containing *Ca*. I. sparus are the more granular cysts (Figure 1c) and the *Ca.* I. hellenicum cysts have the smoother appearance with HE staining. Deconvolution of high resolution confocal FISH images again shows the individual bacterial particles within each cyst (Figure 4c and 4d).

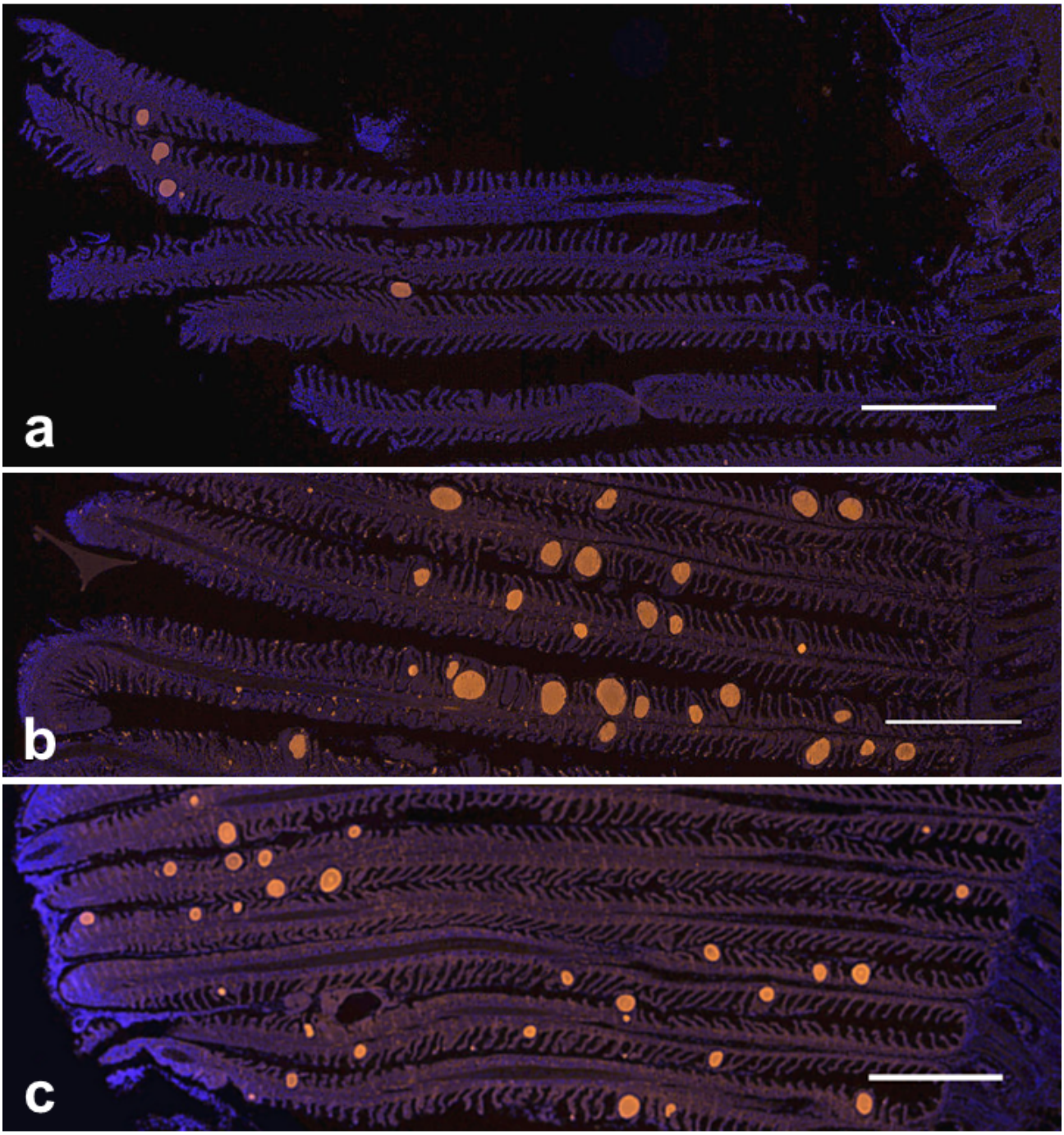

Figure 3 *Images showing the hybridisation of probes designed against novel bacterial sequences to the dominant cyst types. Gill sections from a. 2012Sar3; b. 2013Arg12; c. 2013Ark11. All sections are labelled with Ichthyo290-Cy3 (red) and counterstained with DAPI for DNA (blue). Scale bar in each images represents 400 mm.*

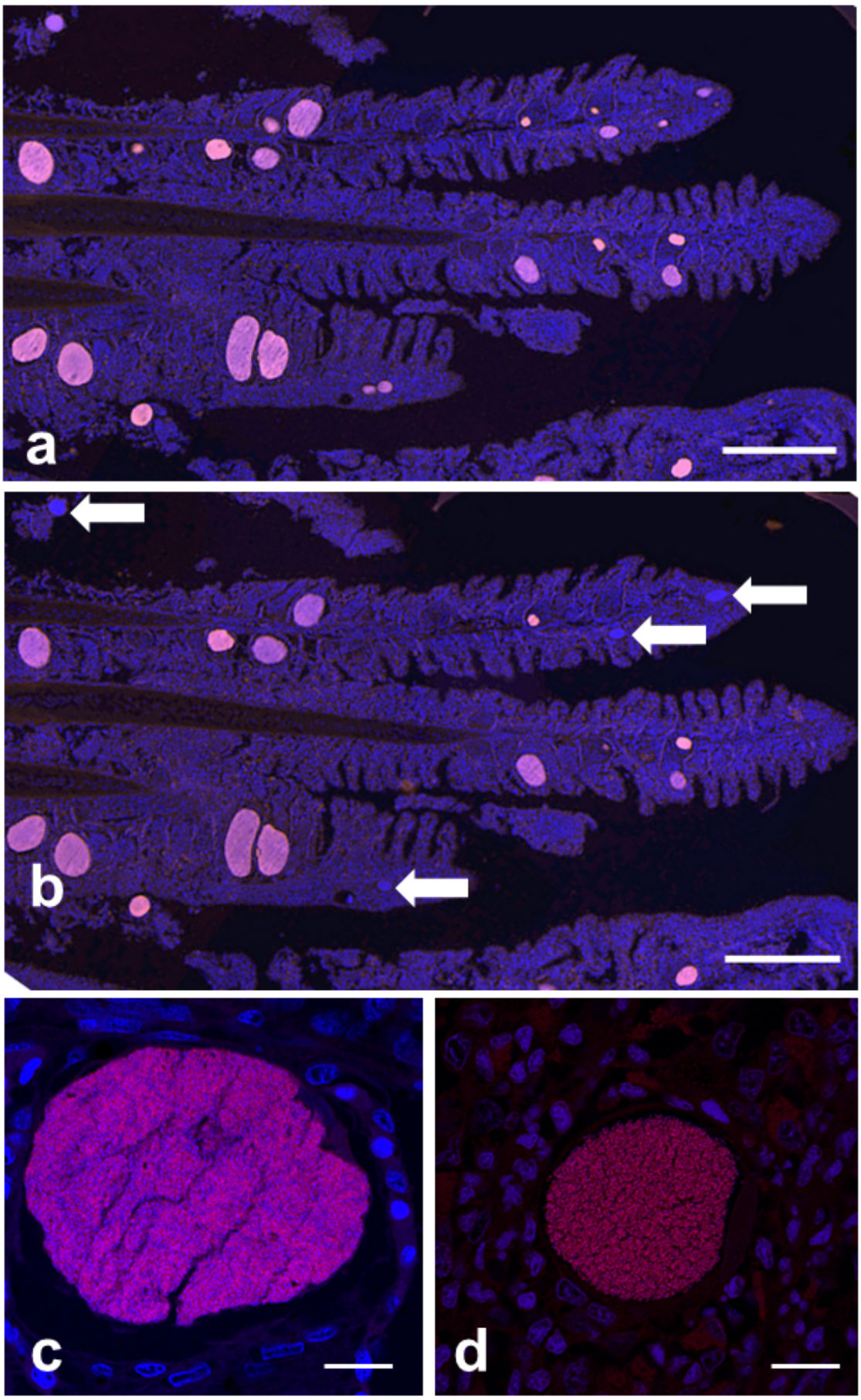
a
b
c
d

Figure 4 *Fluorescent images distinguishing between Ca. I. hellenicum and Ca. I. sparus species. a. and b. Serial sections of fish 2013Arg42 probed using a. Ichthyo290-Cy3 probe against the whole novel genus or b. Ichthyo230-Cy3 probe targeting just Ca. I. hellenicum. Ca. I sparus cysts can be identified as being stained in a. and unstained in b. (arrows). c. and d. Deconvolved images of typical cysts hybridised to probe Ichthyo290-Cy3. c. Ca. I. hellenicum from fish 2013Arg42; d. Ca. I. sparus from fish 2013Arg23. All FISH probes are labelled with Cy3 (red) and all sections are counterstained with DAPI for DNA (blue). Scale bars for a and b are 200 mm, for c and d 10 mm.*

Epitheliocysts are often thought to be associated with chloride cells in gills, which are mitochondrion rich cells heavily involved in osmoregulation and ion transport. We therefore used fluorescent labels to target mitochondria (anti-oxphos complex IV antibody) and chloride cells (anti-sodium-potassium antiporter antibodies) to try to characterise the environs of the cysts. No association was found between cells containing mitochondria, or chloride cells and regions infected with *Ca*. Ichthyocystis (data not shown).

3.4.4 *Ca*. Ichthyocystis species distribution

The two species of *Ca.* Ichthyocystis can be detected and distinguished through FISH and 16S rRNA gene sequencing. We were interested to investigate the geographical and temporal distribution of the species in Mediterranean gilthead seabream. In the 2012Arg and 2012Sar samples with low infection levels, only *Ca*. I. hellenicum can be detected (Table S2). These samples also show a low load of *Ca*. Ichthyocystis by qPCR, and only 1-10 cysts can be observed per section. 2013Arg and 2013Ark samples are from outbreaks of epitheliocystis and are heavily infected. In these gill arches analysed by FISH, both species can often be detected, with cysts containing *Ca*. I. hellenicum more numerous in all samples tested. However, 16S rRNA gene PCR of gill arches from these fish produces sequences from either *Ca*. I. hellenicum or *Ca.* I. sparus, with the latter being more common, and with little correlation with the FISH results (Table S2). As different gill arches from the same fish were used in the different analyses, this indicates that the gill arches may contain different ratios of the two novel species, but that both species are present in the same locations, often coinfecting the same host.

3.4.5 Description of *Ca*. Ichthyocystis epitheliocysts

In order to gain deeper insight into these uncultured bacteria and their interaction with the host tissue, we used further imaging to investigate the cysts. Scanning electron microscopy (SEM) of

samples from Saronikos in 2012 show cysts bulging through the epithelium (Figure 5a, b), with hyperplastic gills and fused lamellae (Figure 5a) coated in an excess of mucus (Figure 5b).

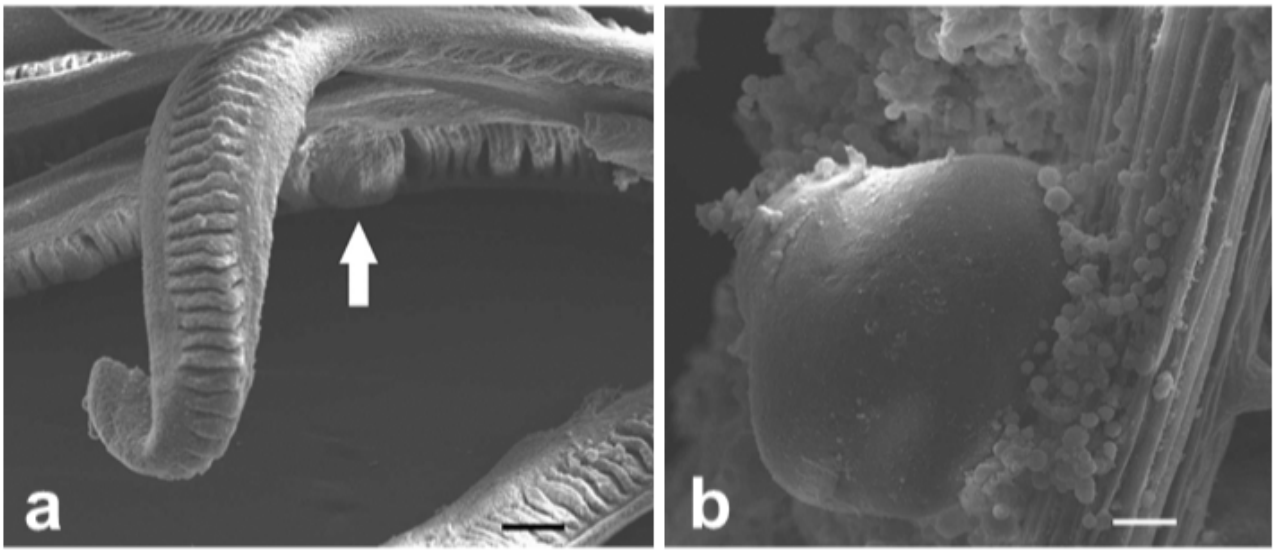

Figure 5 *SEM images of infected gills from Nov 2012 Saronikos. a. Gill filaments showing hyperplasia. A cyst is indicated (arrow). Scale bar represents 100 mm. b. Cyst emerging and surrounded by mucus. Scale bars represent 10 mm.*

TEM was performed on sections from 14 fish (Table S2). From EM images we cannot tell whether we are viewing cysts containing *Ca*. I. hellenicum or *Ca*. I sparus, although *Ca*. I. hellenicum was more commonly found by FISH in these samples. Three-dimensional imaging, using FIB-SEM, allowed further insights, through specific analysis of the sample in any chosen plane which aids the visualisation of specific structures.

There is no evidence of a *Chlamydia*-like developmental cycle, as similar bacterial forms are seen throughout the cyst (Figures 6a, 6b). Epitheliocystis, particularly through its association with chlamydial agents, is assumed to involve intracellular cyst formation; but with the discovery of new, beta-proteobacterial agents, we wanted to determine whether this is still the case. The membranes surrounding the cysts seen under histological (Figure 1d) and concanavalin A (Figure 7a) staining for host cell membranes can be seen in EM images to consist of interdigitating epithelial cell processes, with cell membranes tightly connected by desmosomes (Figures 6b, 7b and Supplementary video file V1). Between the bacteria and the cytoplasm of the host cell there is no membrane barrier visible, suggesting that the cyst lies intracytoplasmically. We were not able to conclusively determine whether the cyst is intracellular within a single epithelial cell which then

becomes enveloped by additional cells, or whether multiple epithelial cells combine to enclose the developing cyst.

The bacteria can be seen to be relatively round with a diameter of 0.5 mm and a length of 0.7 mm (Figures 6c, 6d, 7c). A double membrane surrounds each bacterium (Figures 6d and 7d), which may bud off into the small vesicles which are found in the cyst matrix between bacteria (Figures 6c, 6d and 7d). However, four forms of the bacteria are visible: round with a centrally located dense nucleoid (Figure 6c, 6d, 7b, 7d), although in all 3D-SEM images, the “round” bacteria of 2D images could be shown to be merely cross-sections of the longer forms. Many bacteria were in the process of dividing, often presenting as nucleoids connected by a thin bridge (Figure 6c, 7c) or as long bacteria (Figure 7e) and those with a darker cytoplasm and presenting small clear vacuoles (Figure 6c, 6d, 7f). The specific use of FIB-SEM allowed visualisation of many dividing bacteria possibly indicating rapid growth of the cyst (Supplementary video file V2). Some bacteria also carry an additional dense body, separate from the putative nucleoids (Figure 7c).

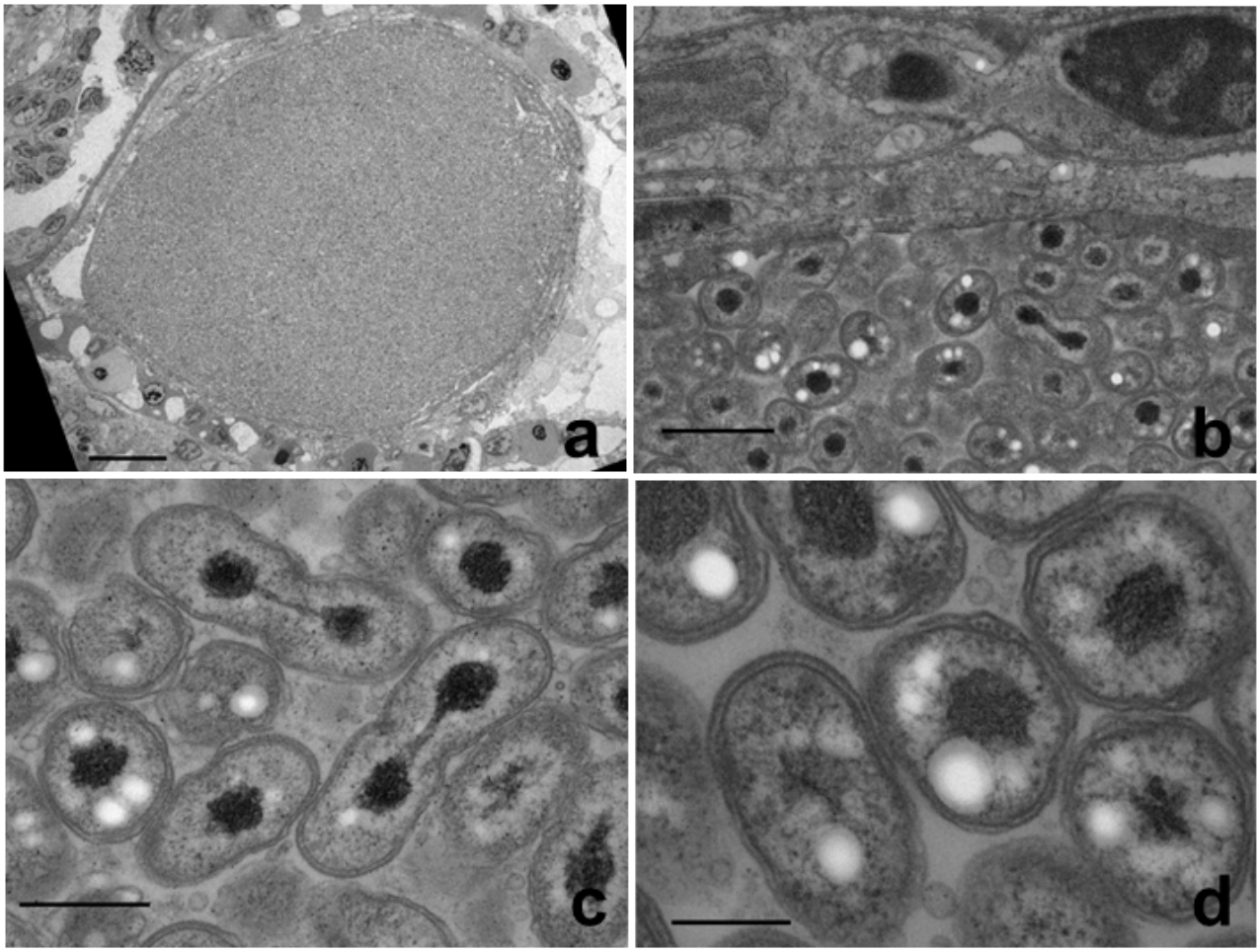

Figure 6 *Representative TEM images from Ichthyocysts from Argolida 2013. a. Overview of cyst from fish 2013Arg14, anchored to the primary lamella and embedded between two secondary*

lamellae, with nucleated erythrocytes visible in the capillary running up the centre of the secondary lamellae. b. Edge of a cyst from fish 2013Arg13, showing the interdigitated epithelial cells, with no membrane visible separating the bacteria from the cytoplasm. c. Dividing bacteria within a cyst from fish 2013Arg13, with the partitioning DNA still connected. Numerous vesicles are present between the bacteria. d. High resolution images from fish 2013Arg12 bacteria, showing the bacterial double membrane, between which rows of periplasmic small electron dense particles can often be seen. Large electron lucent vesicles within the bacterial cytoplasm are common. Also in this image, multiple vesicles in between bacteria are visible. Scale bars represent in a 10 mm, b 1 mm, c 0.5 mm and d 0.3 mm.

Sample	*Ca*. species	Est. genome size	# scaffolds	Largest scaffold	Mean coverage	G+C content	Est. # CDSs	Est. mean CDS length
2013Ark11	I. hellenicum	2.14 Mb	87	272 Kb	215x	35.6	1565	1127 bp
2013Arg41	I. sparus	2.50 Mb	170	254 Kb	549x	38.4	1759	1083 bp
2013Ark22	I. sparus	2.44 Mb	111	280 Kb	190x	38.4	ND	ND
2013Ark32	I. sparus	2.34 Mb	86	288 Kb	220x	38.5	ND	ND

Table 2 *Features of assembled Ca. Ichthyocystis genomes. CDS, coding sequence. ND, Not determined.*

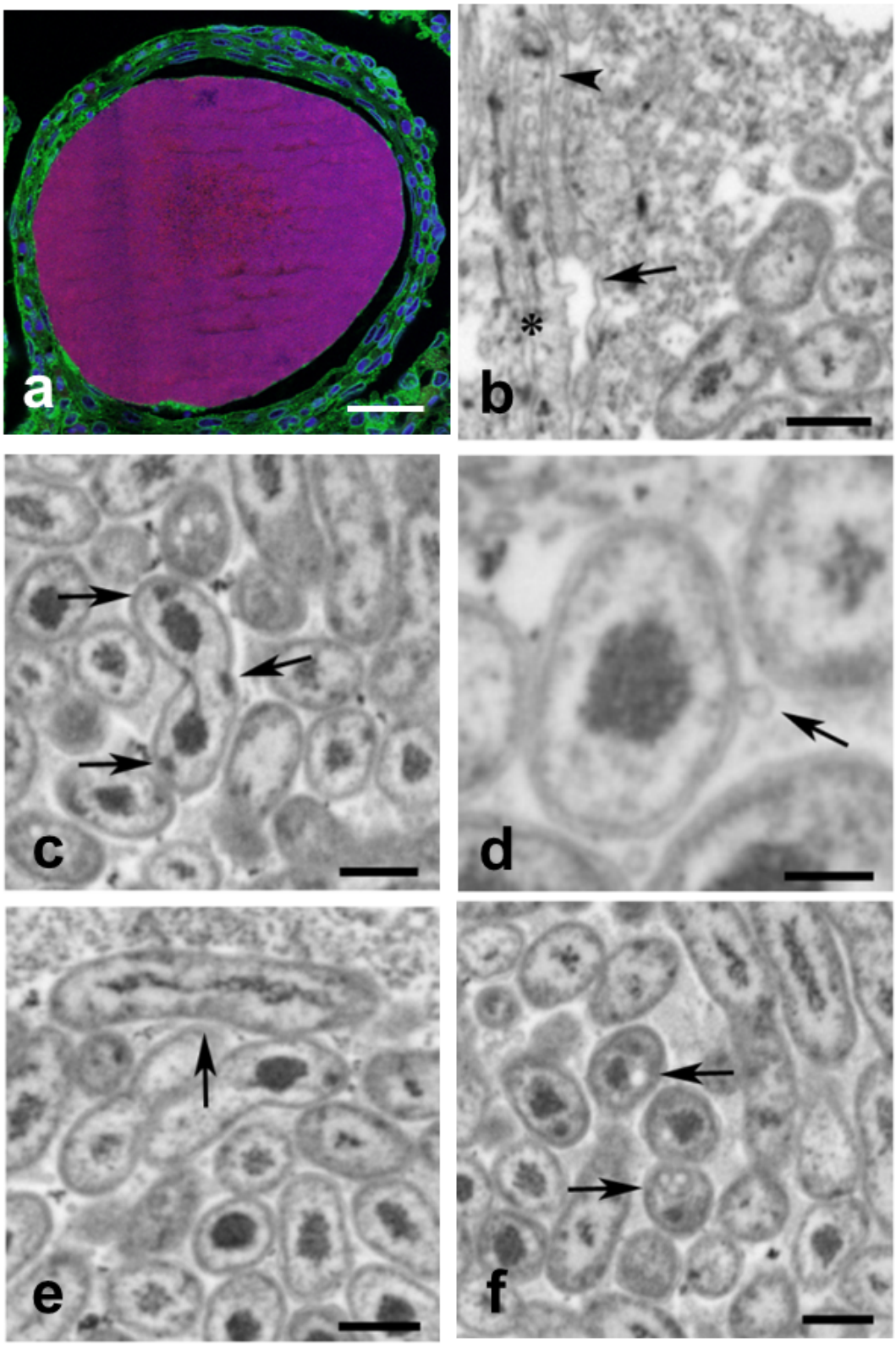

Figure 7. Images selected from FISH and FIB-SEM 3D data of a cyst from fish 2013Arg42. a. *Ca.* I. hellenicum from fish 2013Arg23 hybridised with Ichthyo-290 (red) and subsequent Alex488-concanavalin A staining (green) for glycoprotein membrane proteins, mainly of plasma membranes, counterstained with DAPI for DNA (blue). Membranes can be seen completely

enveloping the cyst. The wave-like patterns within the cyst result from sectioning artefacts. **b**. Edge of a cyst showing interdigitating epithelial cells (arrows) and desmosomes connecting cell processes (asterisk). **c.** A dividing bacterium (centre). Some bacteria contain a dense body not connected to the major body of suspected nuclear material (arrows). **d.** The clearly defined double membrane is visible, connected to small vesicles (arrow). **e**. A long bacterium is indicated (arrow). **f.** Small clear vacuoles are present in many bacteria (arrows). Scale bars in a: 20 mm; b, c, e and f: 500 nm; d: 200nm. Videos of the 3D data of Figure 7b and 7c are presented in Supplementary Files V1 and V2.

3.4.6 Genome of *Ca*. Ichthyocystis

Genomes were obtained directly from heavily infected material without culture (2013Arg and 2013Ark samples stored in RNALater or ethanol). Ten samples of DNA extracted from micromanipulated individual or pooled cysts, was quantified and subjected to whole genome sequencing before or after whole genome amplification (WGA) (Table S2). The resulting sequence data was screened against a database of candidate epitheliocystis bacterial 16S rRNA genes to determine the source of the sequenced DNA and estimated genome coverage. All samples mapped to the *Ca*. Ichthyocystis 16S rRNA gene, with no coverage of chlamydial sequences, confirming that these large micromanipulated cysts are the result of infections by members of the *Ca*. Ichthyocystis genus. The host source was confirmed by mapping sequence data against known gilthead seabream sequences.

Two samples were chosen for *de novo* assembly, with the highest estimated coverage, representing *Ca.* I. hellenicum (2013Ark11, est. 130x) and *Ca.* I. sparus (2013Arg41, est. 240x). Scaffolds were removed from the assembly if they were thought to contain sequence from contaminating species or be too small to be informative (see Methods). The genomes can be seen to be relatively small for beta-proteobacteria at approx. 2.3 Mb (Table 2; mean beta-proteobacterial genome size 5.1 Mb, range 0.2-9.7 Mb).

By comparing the assemblies, contigs were rearranged to determine a putative shared core genome for the genus (Figure S4). The genomes of *Ca.* I. hellenicum and *Ca.* I. sparus share regions comprising approximately 1.5 Mb (65-69% of the full assemblies respectively) with approximately 70% average nucleotide identity (ANI), which is represented by 17-20 scaffolds in the two samples. As such, they can be defined as separate species, using the genomic definition that members of the same species should share over 69% genome coverage at 95% ANI (Goris et al., 2007). Furthermore, using a percentage of conserved proteins (POCP) method with 50% cutoff

to delineate genera (Qin et al., 2014), these two novel species are defined as the same genus, sharing 56.6% of their coding sequences (CDSs).

The remaining sequenced samples with lower coverage were mapped against the core genes: the comparative phylogeny mirrors the 16S rRNA gene phylogeny and gives an indication of the diversity of this genus within Mediterranean epitheliocysts (Figure S5). After mapping back to the assembled genomes, not all the samples were found to contain homogeneous single nucleotide polymorphisms (SNPs). As most of the samples prepared for sequencing contained multiple cysts, it seems that these heterogeneous SNPs may represent variable site in mixed strains of *Ca.* Ichthyocystis, and this even applies to the two samples which were initially assembled, 2013Ark11 and 2013Arg41. The phylogenetic analysis may be confounded by the heterogeneous SNPs from these mixed populations and therefore Figure S5 should only be used as an approximate indicator of diversity. However, three *Ca.* I. sparus samples with high coverage contain only homogeneous SNPs: 2013Arg14, 2013Arg22 and 2013Arg32, the latter two genomes having been sequenced from single cysts. This provides a preliminary indication that single cysts are caused by single bacterial strains. These genomes also display diversity within the *Ca.* I. sparus species. The sequence data from the latter two samples were also assembled, to represent this diversity within the species, and also confirm the approximate genome size of *Ca.* Ichthyocystis (Table 2).

The most prominent feature of the genomes of these species is the occurrence of expanded families of related genes, first identified as large regions of difference between the genomes of the two species. Gene families were initially identified through manual curation of the genome annotations of 2013Ark11 and 2013Arg41, after which family specific HMMs were built and used to re-scan these genomes, with the aim of identifying as many family members as possible. In this way we have identified a total of 433 (28% of total annotated CDSs) and 544 (31%) CDSs in the two genomes respectively, grouped into 30 families, of which 9 are unique to *Ca*. I. hellenicum, 9 to *Ca*. I. sparus, and 12 have members present in both species. Many of these genes occur within tandem arrays within the current assembled scaffolds. Within any strain, between 1 and 200 members of any family has been identified, indicating that the duplication and diversification of these families is a significant evolutionary strategy in this genus. The HMMs generated have also been used to screen several protein databases, yet still very few of these families have any putative function assigned to them; one which contains up to 96 family members in the *Ca*. I. sparus genome, but is absent from *Ca*. I. hellenicum, contains the Pfam PF07906 domain related to the ShET2 enterotoxin N terminal domain. Members of this family were also identified within the draft genomes of 2013Arg22 and 2013Arg32, phylogenetic analysis of which showed that many of

the family members were shared by all three *Ca*. I. sparus genomes, while strain-specific gene expansion was also observed.

No mobile elements were identified within these genomes, in the form of insertion sequences or phages, and very few pseudogenes have yet been identified. Automated annotation also identifies CDSs putatively involved in Type II and Type III secretion, and peptidoglycan synthesis. Putative Major Facilitator Superfamily (MFS) transporters have been found in both species, although there is no clear evidence of the acquisition of specifically antibiotic resistant genes within this genus. Further analysis of the large gene families and also the core genome is ongoing.

3.5 Discussion

We have identified and characterised a novel emerging pathogen of the economically important aquaculture species, gilthead seabream. While epitheliocystis has been periodically described in this fish species, the increase in incidence and mortality associated with this disease is a current cause for concern [Dr Kantham Papanna, personal communication]. Although chlamydial organisms have been found to be responsible for the vast majority of previous cases of epitheliocystis, we describe previously unknown beta-proteobacteria as the major causative agents in fish farms around Greece. This study represents the most thorough investigation of this disease yet, using advanced microscopy and genomics to full advantage to describe the nature of this uncultured bacterial genus. Two distinct species, *Ca.* Ichthyocystis hellenicum and *Ca*. I. sparus, are found co-circulating during heavy infections, and we have provided an insight into the diversity within and between these species.

From 16S rRNA gene sequence, we identified the pathogens as a novel genus of beta-proteobacteria, most closely related to an organism identified within epitheliocysts of Atlantic salmon (*Salmo salar*) in Norway and Ireland (Mitchell et al., 2013; Toenshoff et al., 2012). Further to this we used genomic data, obtained from infected gill material, to confirm the identity of the dominant infectious organisms and prove the novelty of these bacteria. Four genomic sequences have been assembled, two from single epitheliocysts, and a further five have been analysed by mapping. The nine genomes provide information on the diversity of these bacteria, and indicate that there may be many further strains involved in epitheliocystis in the Mediterranean left to explore.

Initial analysis of the gene content within this genus shows that the two species share only 65-69% of their genome, representing the core genes. The small genome size and paucity of identified pseudogenes indicates at first glance that these are compact, highly adapted genomes, consistent with an intracellular lifestyle (Merhej et al., 2009). The regions unique to each species show a

fascinating feature, of massive duplication and diversification of several gene families with unknown function. While horizontal gene transfer has been found to be the larger influence in gene family expansion in most genomes studied to date (Lerat et al., 2005; Treangen and Rocha, 2011), the lack of mobile elements and the tandem locations of these paralogues indicate that these more likely arose from gene duplication and subsequent mutation. Such a system of genome diversification has been previously noted within pathogens, such as the variable surface lipoproteins Vsp family in *Mycobacterium bovis* (Nussbaum, 2002) and Vlp family in *Mycoplasma hyorhinis* (Citti et al., 2000), and appears to be a mechanism commonly used by obligate intracellular bacteria, with examples including the polymorphic membrane proteins within *Chlamydia* species (Stephens et al., 1998; Thomson et al., 2005), effector proteins in the related species *Protochlamydia* and *Neochlamydia* (Domman et al., 2014), and several gene families in *Ehrlichia ruminantium* (Collins et al., 2005). However, the scale of this within the genomes of *Ca.* Ichthyocystis, with the gene families comprising over a quarter of the CDSs annotated, would appear to be unprecedented. Further studies are essential to investigate the functions of these paralogues, and whether it is possible to relate phenotypic differences to the alternative gene families.

This work presents the first genomes of epitheliocystis-causing bacteria to be published, and the draft genomes will be improved, annotated and published in their own right. The relatively small genome is comparable in size to that of many members of the obligate intracellular Chlamydiae and it will be intriguing to see whether further parallels can be found when the first chlamydial epitheliocystis genome becomes available. Future analysis will provide insights into the metabolic capacity, host adaptation, virulence and genome dynamics of this genus. Culture would be essential to generate a complete genome of this bacterium and this is something that we shall continue to explore.

We have shown the power of the use of FISH in correctly identifying causative agents of disease. The targeting of 16S rRNA genes from previous candidate bacteria (Chlamydiae) in the absence of further work would have resulted in false identification of these pathogens. Using fluorescent imaging, EM and the latest techniques in 3D electron microscopy we have been able to characterise these novel bacteria in much greater detail than is normally possible for uncultured species.

EM shows that the bacteria are intracellular, contained either within a single epithelial cell or a construct built up of multiple cells. In either case, the encapsulation by overlapping membranes from surrounding cells, themselves connected by multiple desmosomal-like structures, indicates the cysts are likely to be separated from the outside environment by epithelial cells. The

recruitment and reorganisation of the epithelial cell surround is presumably controlled by the bacteria, which would permit the controlled and efficient supply of cellular nutrients, necessary for the rapid cyst growth evidenced by the apparent constant division of bacteria throughout the cysts. How this is achieved must await further investigations which, for the first time in research into epitheliocystis, can be driven by genomic data. Around intact as well as ruptured cysts we also see macrophages and neutrophils indicating that they are not completely shielded from the host immune system. This immune reaction may lead to resolution of the infection, and protection of the host against subsequent infection.

The reason for the increasing incidence of epitheliocystis is still an open question and a cause for considerable concern. Several of the features in the *Ca*. Ichthyocystis EM images are shared with those in previous cases of gilthead seabream infections since 1975 (Crespo et al., 1999; Paperna, 1977; Paperna et al., 1978; Paperna et al., 1981), in particular the different forms corresponding to proposed stages in the pathogen developmental cycle (Crespo et al., 1999), indicating that these pathogens may have been circulating, unidentified, for decades. The first characterisation of this disease in this host was within the Gulf of Eilat in 1975 (Paperna, 1977), indicating a possible effect of the Suez channel in Lessepsian migration of non-native species from the Red Sea to the Mediterranean. The role of increasingly intensive aquaculture practices should also not be ignored, and the part played by animal husbandry (Segner et al., 2011). Epitheliocystis may also increase the effects of deleterious co-infections, meaning that a vaccine against these bacteria could be of interest to the aquaculture industry: inoculations against vibriosis and pasteurellosis are already routinely performed. It is essential to understand the diversity of these infectious agents in order for a successful vaccine to be developed. In the work presented here, we have made a critical first step towards this goal.

3.6 Taxonomy

"*Candidatus* Ichthyocystis hellenicum" and "*Candidatus* Ichthyocystis sparus" spp. nov., recovered from gilthead seabream (*Sparus aurata*). Ichthyocystis (Ich'thy'o.cy'stis. Gr. masc. n. ichthys [ikhthýs], fish; N.L. fem. cystis from Gr. fem. n. kustis, bladder; N. L. fem. N. Ichthyocystis, cyst of fish), hellenicum (Hel'le'nic.um. N.L. neut. hellenicum, from Greece), sparus (Spa'rus. N.L. n. –a zoological genus name of the fish host). The 16S rRNA gene sequences of the new species place them within a novel bacterial genus. Beta-proteobacteria causing cysts within fish gills, not cultivated. Cysts are membrane-enclosed, staining basophilic under haematoxylin and eosin, and react with an intense and specific labelling when a FISH probe designed to be specific to *Ca.* Ichthyocystis is applied.

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Chapter 4

A zebrafish larval infection model for Chlamydia: the obligate intracellular pathogen *Waddlia chondrophila*

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Planning the experiments, bath-immersion and microinjection experiments, bacteria and cell culture, immunofluorescence stainings, confocal microscopy and image analysis, quantitative PCR, morpholino knockdown and Western Blot, statistical analysis, preparation of the figures, writing of the manuscript

4.1 Abstract

Bacteria belonging to the phylum Chlamydiae are important pathogens of humans and animals, yet many features of the pathogenesis and host specificity are still unknown. This is particularly true for chlamydial species able to utilise either epithelial cells or phagocytic cells as *in vitro* hosts. It has been postulated that phagocytic cells such as macrophages could act as “Trojan host cells”, taking up and aiding the dissemination of these Chlamydiae in the animal host. One of the few animals permitting us to “look inside” to follow such an event is the transparent zebrafish larva. Here we describe the successful development of the first larval zebrafish model for infections with an obligate intracellular pathogen and member of the Chlamydiae, *Waddlia chondrophila* is an infectious agent of humans and cattle and can grow and replicate in macrophages, amoebae and epithelial cells and is an infectious agent of humans and cattle. In bath immersion experiments, bacteria taken up orally, *W. chondrophila* was able to infect and replicate in the swim bladder epithelium. These finding were well reproducible by microinjection of *W. chondrophila,* which leads to the recruitment of macrophages and neutrophils to the infection site where they could be seen to take up the bacteria. *W. chondrophila* appeared to be able to replicate in these innate immune cells, which may serve as a shuttle to penetrate epithelial and endothelial borders and to disseminate in the organism. Macrophage and neutrophil levels were significantly depleted upon an initial systemic infection with *W. chondrophila*, consistent with the ability of *W. chondrophila* to lyse phagocytic cells as part of the mechanism for release of infectious particles following replication. MyD88 mediated signalling contributes to the recruitment of innate immune cells upon infection which probably plays a role in a subsequent efficient eradication of *W. chondrophila* by the innate immune system leading to an increased larval survival and reduced bacterial load of larvae during a systemic infection.

4.2 Introduction

The bacterial species *W. chondrophila* is a purported abortifacient pathogen of cattle (Dilbeck-Robertson et al., 2003), first isolated from a cow abortion in the United States (Dilbeck et al., 1990) and subsequently from a similar case in Germany (Henning et al., 2002). The *Waddliaceae* is one of eight families described to date, within the phylum Chlamydiae (Collingro et al., 2011), all of which are obligate intracellular pathogens able to infect a variety of hosts covering much of the animal kingdom. Expanding genomic information has greatly improved our understanding of potential mechanisms underlying this host diversity and organ tropism, however, there is a great need to develop animal models to test the ideas coming from these efforts (Bachmann et al., 2014). Ideally, such an animal model would lend itself to high throughput screening and share an immune system with close similarities to that of humans and other animal hosts. The most well known family of this phylum is the Chlamydiaceae, classical pathogens of humans and animals, some of whom are known for their high zoonotic potential and ability to cross species borders. Two of the oldest and best described examples are *Chlamydia psittaci*, the agent of psittacosis in birds and humans and *Chlamydia abortus*, an agent of foetal death and abortion in ruminants and humans (Longbottom and Coulter, 2003). *W. chondrophila* may similarly pose a zoonotic risk, based on evidence from serological tests and quantitative real-time PCR in cases of human miscarriage and respiratory disease (Baud et al., 2011; Baud et al., 2007; Haider et al., 2008). A marked difference to the Chlamydiaceae, however, is the ability of *W. chondrophila* to infect and replicate in phagocytic cells, including macrophages and free-living amoeba, at least *in vitro* (Goy et al., 2008). Knowledge of the mechanisms and routes of *W. chondrophila* infection *in vivo* is largely speculative, mainly due to the difficulty of tracing these processes in whole animals. This knowledge is, however, key to the design and application of effective treatment strategies.

The first cultivation of *W. chondrophila* was achieved in bovine turbinate cells and mouse macrophages (Dilbeck et al., 1990; Kocan et al., 1990). Subsequent *in vitro* studies showed that *W. chondrophila* is able to infect and replicate in McCoy cells, buffalo green monkey cells, human fibroblasts (Henning et al., 2002), Vero cells, human pneumocytes and endometrial cells (Kebbi-Beghdadi et al., 2011b), as well as in human macrophages (Goy et al., 2008). During the infection of macrophages *W. chondrophila* avoids degradation by successfully preventing the fusion of the endosome with a lysosome (Croxatto and Greub, 2010). Freshwater amoebae of the genus *Acanthamoeba* are also susceptible to infection with *W. chondrophila* (Lamoth and Greub, 2010). More recently *W. chondrophila* was found to be able to invade and proliferate in the two fish cell lines derived from fathead minnow (*Pimephales promelas*; EPC-175) and from rainbow trout (*Oncorhynchus mykiss*; RTG-2) (Kebbi-Beghdadi et al., 2011a). In addition to mammalian hosts,

W. chondrophila has been isolated from aquatic environments as diverse as sediments from the eastern Mediterranean Sea (Polymenakou et al., 2005) and freshwater samples from well water sources in Spain (Codony et al., 2012). According to these findings it has been speculated that freshwater protists and fish could potentially serve as an aquatic reservoir for *W. chondrophila* and that one possible transmission route is water-borne.

Indeed, chlamydial disease affects both marine as well as freshwater fishes causing the disease epitheliocystis (Draghi et al., 2004; Hoffman et al., 1969; Meijer et al., 2006) in which bacteria-filled intracellular inclusions are found infecting gill and skin epithelia. Several chlamydial agents of epitheliocystis have been described so far, some of which are closely related to the Waddliaceae such as *Ca.* Clavochlamydia salmonicola (Clavochlamydiaceae) (Karlsen et al., 2008) and *Ca.* Syngnamydia venezia (Simkaniaceae) (Fehr et al., 2013), whereas others are more diverse such as *Ca.* Piscichlamydia salmonis (Piscichlamydiaceae) (Draghi et al., 2004). The question therefore arises, could members of the Waddliaceae directly infect fish and could the zebrafish be the model vertebrate host for Chlamydia we are looking for?

As a model organism in infection biology the zebrafish (*Danio rerio*) has become increasingly popular. Many infection systems using larval and adult zebrafish have been successfully developed over the past decade (reviewed in (Kanther and Rawls, 2010; Meijer and Spaink, 2011)). Its small size, ease of breeding, high fertility and genetic tractability combined with transparent larval stages combine to make it an attractive model organism for science. The zebrafish immune system displays many similarities to that of mammals, with counterparts for most of the human immune cell types (reviewed in (Meeker and Trede, 2008)). The zebrafish innate immune system starts to develop as early as 24 hours post fertilisation (hpf) with a population of primitive macrophages which derive from cells located in a region of the yolk sac near the heart (Herbomel et al., 1999). At 2 days post fertilisation (dpf) subpopulations of macrophages can already be observed throughout the organism along with neutrophils whose development initiates between 32-48hpf. The development of the adaptive immune system lags behind, with the first lymphocytes observed from 4 dpf, although fully developed adaptive immunity takes another 4 weeks to mature (reviewed in (Meijer and Spaink, 2011)). For this reason, it is possible to exclusively observe the reaction of the innate immune system within an infection during the first week of larval development. Hence, if we could infect zebrafish with *W. chondrophila*, we would have a test system to examine the initial infectious pathways.

Our study also aimed to shed some light on the primary reaction of the innate immune system to a *W. chondrophila* infection. Cells of the innate immune system recognise pathogen-associated molecular patterns (PAMPs) of bacteria with their pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are PRRs that recognise extracellular and endosomal PAMPs such as

lipopolysaccharide, peptidoglycan and bacterial nucleic acids. The activation of PRRs initiates an inflammatory response through signalling cascades that lead to cytokine production which promote recruitment of leukocytes to the infection site and phagocytosis of invading pathogens (reviewed in (Newton and Dixit, 2012)). A key factor for TLR signal transduction is the downstream adaptor molecule myeloid differentiation factor 88 (MyD88). MyD88 interacts with all known TLRs and members of the IL-1R family, linking these receptors to activation of the IRAK complex, resulting in the induction of NF-kB and MAPK signalling ((Medzhitov et al., 1998), reviewed in (Warner and Nunez, 2013)). Previous studies have shown that recognition by Toll-like receptors could mediate an efficient immune reaction against chlamydial infection leading to bacterial clearance (Naiki et al.) while in other cases Toll-like receptor dependent recruitment of innate immune cells had an adverse effect by enhancing the bacterial load during an infection (Rodriguez et al.). The function of the TLR and its downstream signalling cascades, including MyD88, is well conserved from zebrafish to humans (Hall et al., 2009). So we decided to investigate the impact of Toll-like receptor recognition on the immune reaction to *W. chondrophila* in knockdown larvae with depleted levels of the signalling molecule MyD88.

Zebrafish larvae have been used to study infections of many different bacterial pathogens like *Mycobacterium marinum*, *Salmonella typhimurium*, *Vibrio anguillarum*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Staphylococcus aureus*, *Streptococcus iniae* and *Shigella flexneri* (Brannon et al., 2009; Clatworthy et al., 2009; Davis et al., 2002; Harvie et al., 2013; Herbomel et al., 1999; Levraud et al., 2009; Mostowy et al., 2013; O'Toole et al., 2004; Prajsnar et al., 2012; van der Sar et al., 2003; Vergunst et al., 2010). We have studied infection with *W. chondrophila*, developing the first infection model for an obligate intracellular pathogen and member of the *Chlamydiae* in zebrafish larvae.

4.3 Materials and Methods

4.3.1 Zebrafish strains and husbandry

Zebrafish (*Danio rerio*) strains used in this study were predominantly *albino* mutants (slc45a2$^{b4/+}$) and transgenic fish of the *Tg(lyz:DsRED2)nz50* line that produce red fluorescent protein in cells of the myelomonocytic lineage able to migrate to inflammatory sites and phagocytose bacteria (Hall et al., 2007), primarily neutrophils from 50 hpf (ref: Clatworthy et al., 2009). In addition, the *Tg(fli1a:eGFP)* line which produces green fluorescent protein in endothelial cells, was also used for visualising the vascular system (Lawson and Weinstein, 2002). Adult fish were kept at a 14/10 hours light/dark cycle at a pH of 7.5 and 27°C. Eggs were obtained from natural spawning between adult fish which were set up pairwise in separate breeding tanks. Embryos were raised in petri

dishes with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) containing 0.3 μg/ml of methylene blue at 28°C. From 24 hpf, 0.003 % 1-phenyl-2-thiourea (PTU) was added to prevent melanin synthesis. The *albino* mutants lack melanised melanophores, and for these PTU treatment was not necessary. Staging of embryos was performed according to Kimmel et al. (Kimmel et al., 1995).

Research was conducted with approval (no. 216/2012) from the Veterinary Office, Public Health Department, Canton of Zurich (Switzerland). Larvae were maintained up until 8 dpf, at which time all were euthanized by applying an overdose of 4 g/l buffered tricaine (MS-222, Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich) in accordance with ethical procedures.

4.3.2 Bacterial cultures

W. chondrophila strain WSU 86-1044 (ATCC VR-1470) and *Acanthamoeba castellanii* strain ATCC 30010 were kindly provided by Professor Gilbert Greub, CHUV (Lausanne, Switzerland). *W. chondrophila* were grown within amoebae in 25 cm^2 cell culture flasks containing 10 ml of peptone yeast extract glucose (PYG) broth (Greub and Raoult, 2002). Cultures were harvested after 5 days and filtered through a 5 μm membrane to remove remaining amoebae. The flow-through was centrifuged at 7000 g for 15 min. The resulting pellet of bacterial elementary bodies (EBs) was then suspended in E3 medium for bath immersion or PBS for microinjection experiments. Inclusion forming units (IFU) of the culture were determined by infecting monolayers of EPC cells in 24-well plates with a 10-fold dilution series of 1 μl of the bacterial suspension at 28°C. After 24 h cells were fixed with 4 % paraformaldehyde and subsequently stained with a primary rabbit anti-waddlia antibody and detected with a secondary goat anti-rabbit-IgG conjugated to a fluorescent AlexaFluor dye. After imaging with a fluorescent microscope, inclusions were counted in Imaris (Bitplane) to calculate a mean IFU value.

4.3.3 Bath immersion experiments

Zebrafish embryos between 24 hpf and 4 dpf were incubated in groups of 15 for each time point and each condition in 24-well plates with E3 medium containing 2 x 10^9 IFU/ml of *W. chondrophila* at 28°C. Embryos younger than 48 hpf were manually dechorionated prior to immersion. After 4 hours of incubation embryos were washed twice in fresh E3 medium and transferred to 6-well plates containing 4 ml of E3 medium per well and further incubated at 28°C. Embryos were then observed under a binocular microscope for signs of disease and survival twice a day. At several time points embryos from each group were euthanized in E3 medium containing 4 mg/ml buffered tricaine (MS-222).

4.3.4 Microinjection experiments

For microinjections of *W. chondrophila* into zebrafish larvae bacteria were first harvested from a 5 days old amoebal co-culture as described above. The concentration of the *W. chondrophila* EBs was adjusted to 1000 to 2000 IFU/nl in PBS and 0.085 % phenol red was added to visualise the injection procedure. Injections were done using borosilicate glass microcapillary injection needles (Science Products, 1210332, 1 mm O.D. x 0.78 mm I.D.) and a PV830 Pneumatic PicoPump (World Precision Instruments). Prior to intravenious injections embryos of 2 dpf were manually dechorionated and anesthetised with 200 mg/l buffered tricaine (MS-222). Afterwards embryos were aligned on an agar plate and injected with 1 nl of the *W. chondrophila* suspension into the Duct of Cuvier, also known as common cardinal vein. The volume of the injected suspension was previously adjusted by injection of a droplet into mineral oil and measurement of its approx. diameter over a scale bar. For swim bladder injections 4 dpf larvae were treated similarly but dechorionisation was not necessary because larvae were already hatched. 4 dpf larvae were then injected with a volume of 2 nl into the lumen of the swim bladder. After injections infected larvae were allowed to recover in a petri dish with fresh E3 medium for 15 min. Subsequently larvae were transferred in 6-well plates in groups of about 15 larvae in 4 ml E3 medium per well, incubated at 28°C and observed under a stereomicroscope twice a day. Samples for IFS, EM and qPCR were taken at 0, 12, 24, 36, 48, 60 and 72 hpi. Sampled larvae were euthanized with an overdose of 4 g/l buffered tricaine and transferred into different buffers and fixatives for subsequent analyses respectively.

4.3.5 Whole mount immunofluorescence and histological stainings

For immunofluorescence (IF), whole zebrafish larvae were fixed in 4% paraformaldehyde at 4°C followed by 100 % methanol overnight at -20°C. Samples were rehydrated in 50 % methanol for 5 min and subsequently in H_2O for 1 h before blocking in PBDT (PBS containing 1 % BSA, 1 % DMSO, 0.5 % Triton X-100, 2.5 % goat serum) for 6-8 h at room temperature. Larvae were then incubated with primary antibody overnight at 4°C. Primary antibody was detected by incubation with a secondary goat anti-IgG antibody conjugated to a fluorescent AlexaFluor dye (Life Technologies) at 4°C overnight. Additionally 4'-6-diamin-2-phenylindole (DAPI) was added to visualise bacterial and host DNA. Stained larvae were prepared for microscopy on objective slides mounted in 1.5 % agarose, 50 % glycerol and screened under a fluorescence microscope. Positive samples were subsequently screened in more detail for *W. chondrophila* inclusions with a confocal laser scanning microscope (CLSM).

For histological examination, whole zebrafish larvae were fixed in 4% paraformaldehyde at 4°C and embedded in cubes of cooked egg white in order to position them correctly for histological sections. These cubes containing the larvae were dehydrated in an ascending alcohol series ending in xylene and afterwards embedded in paraffin. Paraffin blocks were cut in 2-3 μm thin sections, mounted on glass slides and stained using a routine protocol with haematoxylin and eosin (HE).

4.3.6 Light-Microscopy and image analysis

Overview images were performed with an upright light microscope (Olympus BX61) with both bright field and fluorescence modules. The fluorescence filter cube used was optimized for DAPI/FITC/TRIC. For higher resolution images, 3D-image stacks of whole mount samples were prepared using CLSM (Leica TCS SP5, Leica Microsystems). Various combinations of the fluorophors AlexaFluor dyes 594, 546, 488, , GFP, dsRED and DAPI were sequentially excited in descending series with the 594 nm, 561 nm, 488 nm and 405 nm laser lines, with emission signals collected within the respective range of wave lengths. 3D image stacks were collected sequentially (to prevent blue-green–red channel cross-talk) according to Nyquist criteria and deconvolved using HuygensPro via the Huygens Remote Manager v2.1.2 (SVI, Netherlands). Images were further analysed with Imaris 7.6.1 (Bitplane, Zurich, Switzerland) and aligned with Adobe Photoshop Elements 12. Fluorescent cells were quantified with Imaris' fluorescent spot counting tool.

4.3.7 Transmission Electron Microscopy

For electron microscopy, larvae were fixed in a mixed solution of 1 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.5 at 4°C overnight. Afterwards samples were prepared for embedding into epoxy resin and for transmission electron microscopy according to standard procedures. Epoxy resin blocks were screened for larvae by using semithin sections (1 µm) which were stained with toluidine blue (Sigma-Aldrich) to visualise tissue. Ultrathin sections (80 nm) were mounted on copper grids (Merck Eurolab AG, Dietlikon, Switzerland), contrasted with uranyl acetate dihydrate (Sigma-Aldrich) and lead citrate (Merck Eurolab AG) and investigated using a Philips CM10 transmission electron microscope. Images were processed with Imaris (Bitplane) and assembled for publication using Adobe Photoshop.

4.3.8 Quantitative PCR

A qPCR system was designed against the 16S rRNA gene from *W. chondrophila* (forward: 5'-AGTCCGGCTACACCAAGTATGC-3', reverse: 5'-TGGCGAAGGCGGTTTTC-3', probe: 5'-FAM-TTCGCTCCCCTAGCTTTCGGGCAT-TAMRA-3'), allowing quantification of the bacterial load of

individual infected larvae. The TaqMan qPCR system was designed and validated against serial dilutions of amplicons from target and total DNA of non-infected larvae. Serial dilutions of a known concentration of pCR2.1 containing the target sequence were used as standards. Total DNA of individual larvae was extracted with a MagNA Pure LC (Roche) robot and eluted in 100 µl elution buffer. Reactions were carried out on a StepOne Plus real-time PCR system (Applied Biosystems). TaqMan Fast Advanced reagents (Applied Biosystems) were used according to the manufacturer's instructions with 5 µl input DNA in a total reaction volume of 20 µl.

4.3.9 Morpholino knockdown

Knockdown of MyD88 expression was done by standard microinjection of 1 nl of a 5 mM solution of anti-myd88 morpholino (5'-GTTAAACACTGACCCTGTGGATCAT-3', Gene Tools) into the early zygote immediately after fertilisation using borosilicate glass microcapillary injection needles (Harvard Apparatus, 30-0019, 1 mm O.D. x 0.58 mm I.D.) and an Eppendorf FemtoJet. A group of control larvae was injected with 1 nl of a control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3', Gene Tools) with no known target in zebrafish at the same concentration in parallel. Levels of MyD88 protein in morphant and control larvae were analysed by Western Blot at 3, 4 and 5 dpf with a mouse anti-myd88 antibody, detected with a goat anti-mouse-IgG antibody conjugated to horse radish peroxidase (HRP).

4.4 Results

4.4.1 *W. chondrophila* infects the zebrafish swim bladder

In order to first establish whether *W. chondrophila* can infect zebrafish through an oral or dermal route, embryos and larvae of different stages were incubated in a suspension of *W. chondrophila* EBs, in a bath immersion experiment. While younger embryos and larvae of up to 72 hpf stages could not be infected with this method, 4 dpf old larvae appeared to swallow the bacteria and interestingly got an infection in their swim bladder which we detected by immunofluorescence (IF) staining followed by confocal microscopy of whole larvae (data not shown).

Infection via bath immersion carries inherent experimental variability due to varying local concentrations of *W. chondrophila* and different gulping rates of individual larvae. Therefore we tried direct microinjection of 10^3 *W. chondrophila* EBs into the swim bladder lumen of 4 dpf larvae (Fig. 1A), that reproduced the described non-lethal infection of the swim bladder. The resulting infection was comparable to that seen using bath immersion but under more controlled conditions, confirming that directed injection can be used as a more reproducible method of establishing infection. In addition to live *W. chondrophila*, two groups of control embryos were injected with

heat-inactivated *W. chondrophila* or PBS in parallel. Samples were taken at 4, 24, 48, 72 and 96 hours post infection (hpi) and screened for the presence of *W. chondrophila* inclusions by CLSM and TEM. Waddlia inclusions were detected in the epithelium of the swim bladder as well as in adjacent tissues at 24 to 48 hpi (Fig. 1B). In HE staining, the swim bladder walls of infected larvae were markedly thickened when compared with control animals (Fig. 1F,G). The epithelial cells lining the cavity were piled up and were no longer a single cell row and the cells themselves were cuboidal instead of flattened (Fig. 1H). A few neutrophils were found to have migrated into the epithelial cell layers (Fig. 1I). This recruitment of inflammatory cells could be confirmed by using larvae of the transgenic Tg(*lyzC*:dsRed^{nz50}) line, whose neutrophils express red fluorescent protein, easy assessable by fluorescence microscopy. In these larvae, large numbers of neutrophils could be detected clustered inside the swim bladder (Fig. 1E), compared to PBS injected control larvae (Fig. 1D). We further quantified the recruitment by counting the fluorescent neutrophils in Imaris. The results show a significantly increased reaction of neutrophils to live *W. chondrophila* compared to heat-inactivated bacteria (Fig. 1C). The infection appeared to be non-lethal by the end of the observation period of 3 dpi and infected larvae showed no altered behaviour compared to control larvae.

4.4.2 Intravenous microinjection of *W. chondrophila* causes a systemic infection

Because the swim bladder infection remained limited to the epithelium and was only possible to achieve in older larval stages, our next step aimed to produce a systemic infection in younger embryonic stages by intravenous injection. Therefore we injected 48 hpf embryos intravenously via the Duct of Cuvier, also known as common cardinal vein with 10^3 to 10^4 *W. chondrophila* EBs. The injection resulted in a rapid systemic infection with mortalities up to 100 % within the first 24 hours for the highest tested dosage (10^4) and a LD_{50} within 48 hpi of approx. 5×10^3. A dosage of 2×10^3 *W. chondrophila* EBs was chosen for the following experiments to produce moderate mortalities of between 20 and 30 % at 72 hpi. With that dosage larvae showed an increasingly impaired blood circulation between 24 and 48 hpi, resulting in the formation of a pericardial oedema (Fig. 2A). Histologically, no tissue alterations could be seen. With IF imaging, a recruitment of neutrophils and a few macrophages to the vasculature could be observed (video 3).

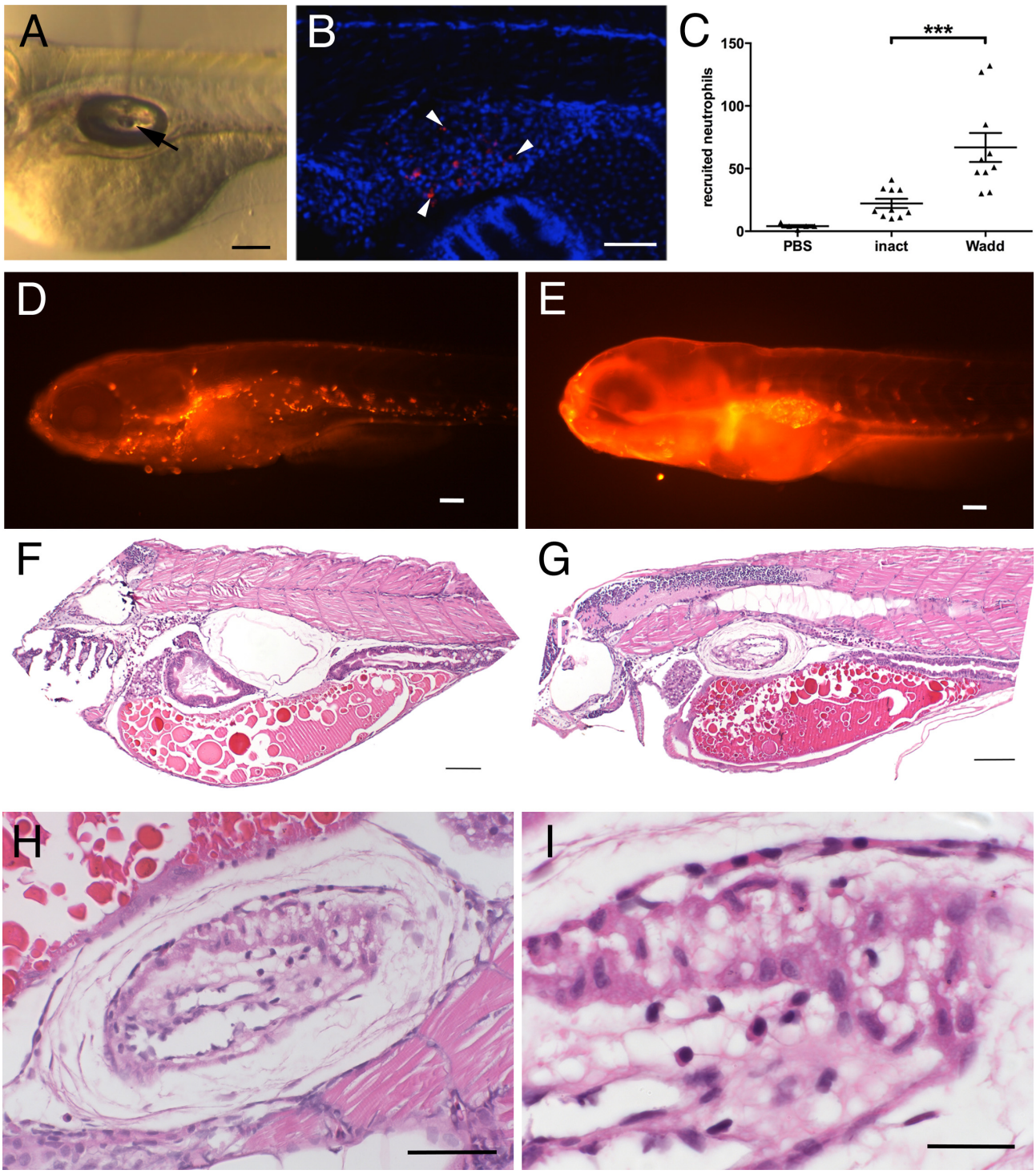

Figure 1 *Swim bladder infection in 4-5 dpf old larvae. (A) Microinjection of W. chondrophila EBs directly into the lumen of the swim bladder of 4 dpf larvae. A drop of approx. 1 nl of the bacterial suspension can be seen hanging on the tip of the injection needle (arrow) inside the air filled lumen of the swim bladder. (B) CLSM acquired 3D stack of the trunk region of a larva at 36 hpi, after IF staining with an anti-Waddlia antibody (red) and DAPI (blue). The swim bladder in the centre of the image exhibits several inclusions inside the epithelium, (arrow heads). (C) The infection provokes*

rapid recruitment of innate immune cells. Recruitment of dsRed expressing neutrophils of the Tg(lyzC:dsRed)nz50 line to the swim bladder can be observed at 8 hpi (E), while PBS injected control larvae show almost no presence of neutrophils in this organ (D). Quantification of the recruitment shows a significantly increased reaction of neutrophils to live W. chondrophila (Wadd) compared to heat-inactivated (inact) bacteria (C). Histology on H&E-stained sections of infected larvae show clear pathological changes in their swim bladder (G), like thickening of the epithelium (H) and infiltration of innate immune cells like macrophages and neutrophils (I), compared to PBS injected control larvae (F). Scale bars (A, B, D, E, F, G) 100 µm, (H) 50 µm and (I) 20 µm.

To follow the distribution of *W. chondrophila* in the vascular system we used the transgenic Tg(fli1a:eGFP) line, which expresses green fluorescent protein in all endothelial cells. Tracking the infection with IF using an anti-*Waddlia* antibody, the maximum number of *W. chondrophila* inclusions were found at 36 to 48 hpi, distributed throughout the whole embryo, with both inclusions located in the vasculature and others found beyond the bounds of the endothelium (Fig. 2D, F, G). Cell types that were identified to be susceptible to *W. chondrophila* invasion during a systemic infection were predominantly endothelial cells and phagocytosing innate immune cells, macrophages and neutrophils. Especially in regions where the blood is flowing more slowly, such as the fine capillary network of the tail muscles and veins, infection of the endothelium was commonly occurring. Cells of the innate immune system were seen to enter the blood stream upon intravenous injection of *W. chondrophila* and to take up *W. chondrophila* EBs, which could then lead to an infection of the respective cell. Infected immune cells could often be observed to degenerate between 24 and 48 hpi (Fig. 2E). By taking up *W. chondrophila* in the swim bladder or in the blood stream and subsequent migration into other tissues, both innate immune cell types contribute to the crossing of epithelial and endothelial borders and further dissemination of *W. chondrophila* in the host.

In order to additionally quantify the bacterial burden during the infection, we developed a specific quantitative PCR targeting the two-copy W. chondrophila 16S rRNA gene. The measurement of bacterial genome copies within total DNA extracts of individual larvae showed a maximum of bacterial replication between 36 and 48 hpi followed by a decrease at 72 hpi. Larvae that survived the first 72 hpi appeared to be able to clear the infection within the next days with a few scattered inclusions remaining but with no further development of disease or increased mortality until the end of the observation period.

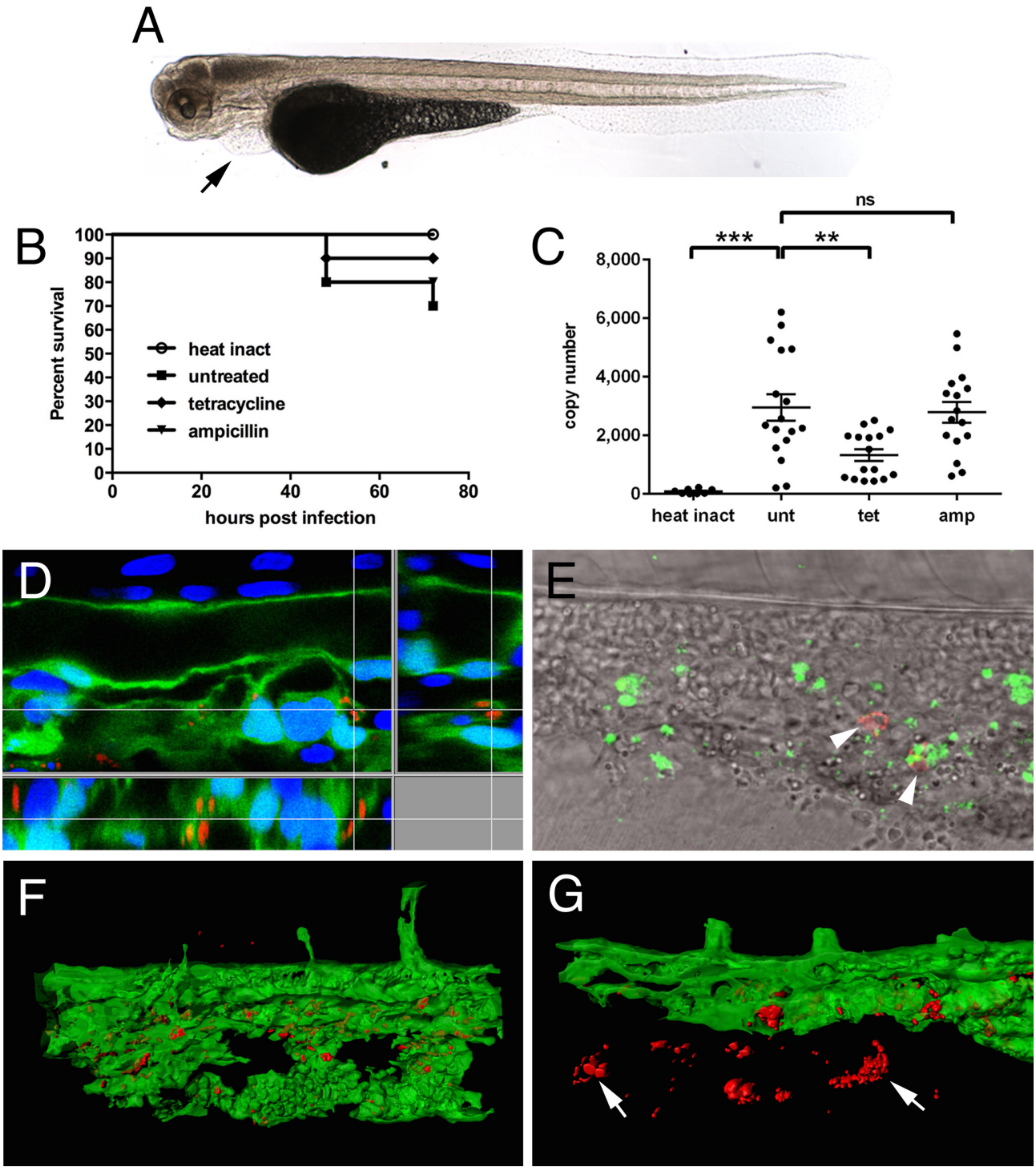

Figure 2 *Systemic infection in 3-4 dpf old larvae. (A) LM appearance of an intravenously injected larva at 48 hpi. With live bacteria injected larvae show at this time point an impaired blood flow and formation of a pericardial oedema (arrow) can be observed. Survival rates (B) and bacterial load (C) at 36 hpi of differently treated larvae after intravenous injection of W. chondrophila and subsequent treatment with 30 µg/ml tetracycline (tet) or 30 µg/ml ampicillin (amp) or left untreated (unt) or injection of heat inactivated W. chondrophila (heat inact). Survival rates of 10 larvae for*

*each condition were observed in three independent experiments, respectively. Bacterial load of individual larvae was determined by qPCR targeting the W. chondrophila 16S rRNA sequence. Statistical analysis was done by one-way ANOVA with Bonferroni's posttest. *** = p < 0.001, ** = p < 0.01, ns = not significant. Mean values ±SEM are shown by horizontal bars. CLSM imaging of intravenously injected transgenic Tg(fli1a:eGFP) larvae after IF staining with an anti-Waddlia antibody shows the infection of endothelial cells (green) with W. chondrophila (red). The perinuclear location inside the GFP expressing endothelial cells can be seen in the section view (D). DNA was stained with DAPI (blue). Surface rendering (with Imaris) of the tail artery and caudal vein shows the distribution of the inclusions inside the vasculature at 24 hpi (F) and outside endothelial bounds (arrows) at 36 hpi (G). Intravenous injection into transgenic Tg(lyzC:dsRed)nz50 larvae shows with W. chondrophila (green) infected and degrading neutrophils (red) in the tail region at 36 hpi (arrow heads).*

4.4.3 Antibiotic treatment of infected larvae

In vitro studies have previously shown that *W. chondrophila* is susceptible to antibiotics of the tetracycline group and resistant to β-lactam antibiotics (Goy and Greub, 2009). Therefore we compared the effect of tetracycline and ampicillin on *W. chondrophila* infections *in vivo*. While treatment with tetracycline significantly ($p<0.01$) reduced *W. chondrophila* replication *in vivo* and increased the survival rate (Fig. 2B, C), treatment with ampicillin had a small but non-significant impact on the bacterial load compared to untreated larvae. Formation and distribution of *W. chondrophila* inclusions in untreated and ampicillin treated larvae were similar. In tetracycline treated larvae inclusion formation was strongly attenuated. Furthermore heat-inactivated bacteria were quickly cleared from the system as shown by qPCR at 36 hpi (Fig. 2C).

4.4.4 Morphology of *W. chondrophila* in zebrafish infections

To investigate the morphological features of *W. chondrophila* infection in zebrafish larvae, we performed detailed TEM and CLSM analyses of infected larvae after IF staining with an anti-Waddlia antibody and an anti-OxPhosIV antibody to stain mitochondria. W. chondrophila could be found to infect different cells of the zebrafish, predominantly epithelial cells of the swim bladder (Fig. 3A, B, E and F), phagocytes of the innate immune system (Fig. 3C, D and G) and endothelial cells (H). The chlamydial inclusions inside those cells exhibited typical features for *Waddlia* infection like the transformation from the smaller metabolically almost inactive EBs with highly condensed DNA, to the larger replicating RB forms inside a BCV. Further, host cell mitochondria

were readily recruited and closely associated with the inclusion, a characteristic for *Waddlia* infection in all determined cell types of the zebrafish. While epithelial and endothelial cells usually contained a single perinuclear inclusion, individual phagocytes could harbour several inclusions of dividing bacteria. In TEM images, some bacteria were in close relation to microvilli on the epithelial cell surface while others were found within the cytoplasm of these cells. The morphological structure of these bacteria on the surface resembled as well the typical morphology known for *W. chondrophila*. The bacteria were round, up to 0.5 μm in diameter, showed condensed DNA in the centre giving the bacterial cell a “target” appearance. Replicating RB’s were enclosed in a bacteria-containing vacuole (BCV). The RB’s were identified by their increased size of 0.9 μm in diameter and the finely distributed chromatin. The perinuclear BCVs were closely associated with host cell mitochondria and endoplasmic reticulum.

4.4.5 *W. chondrophila* infection leads to phagocyte depletion

To further investigate the reaction of the innate immune system we performed again infections within transgenic larvae of the Tg(*lyzC*:dsRed^{nz50}) line. Sterile PBS or live *W. chondrophila* (10^3 EBs) were injected intravenously into 2 dpf larvae, to investigate the impact of a systemic infection on the number of phagocytes. Larvae were sampled at 36 hpi to quantify the total number of innate immune cells. Both, macrophages and neutrophils were quantified after IF staining by counting fluorescent cells in Imaris, with neutrophils detected through LyzC expression and macrophages through Mpeg.1. Both cell types were observed to be recruited to infection sites and to take up *W. chondrophila* particles. At 36 hpi, the numbers of both macrophages and neutrophils were found to be significantly depleted upon a systemic infection with *W. chondrophila* compared to PBS injected larvae (Fig. 4D, E).

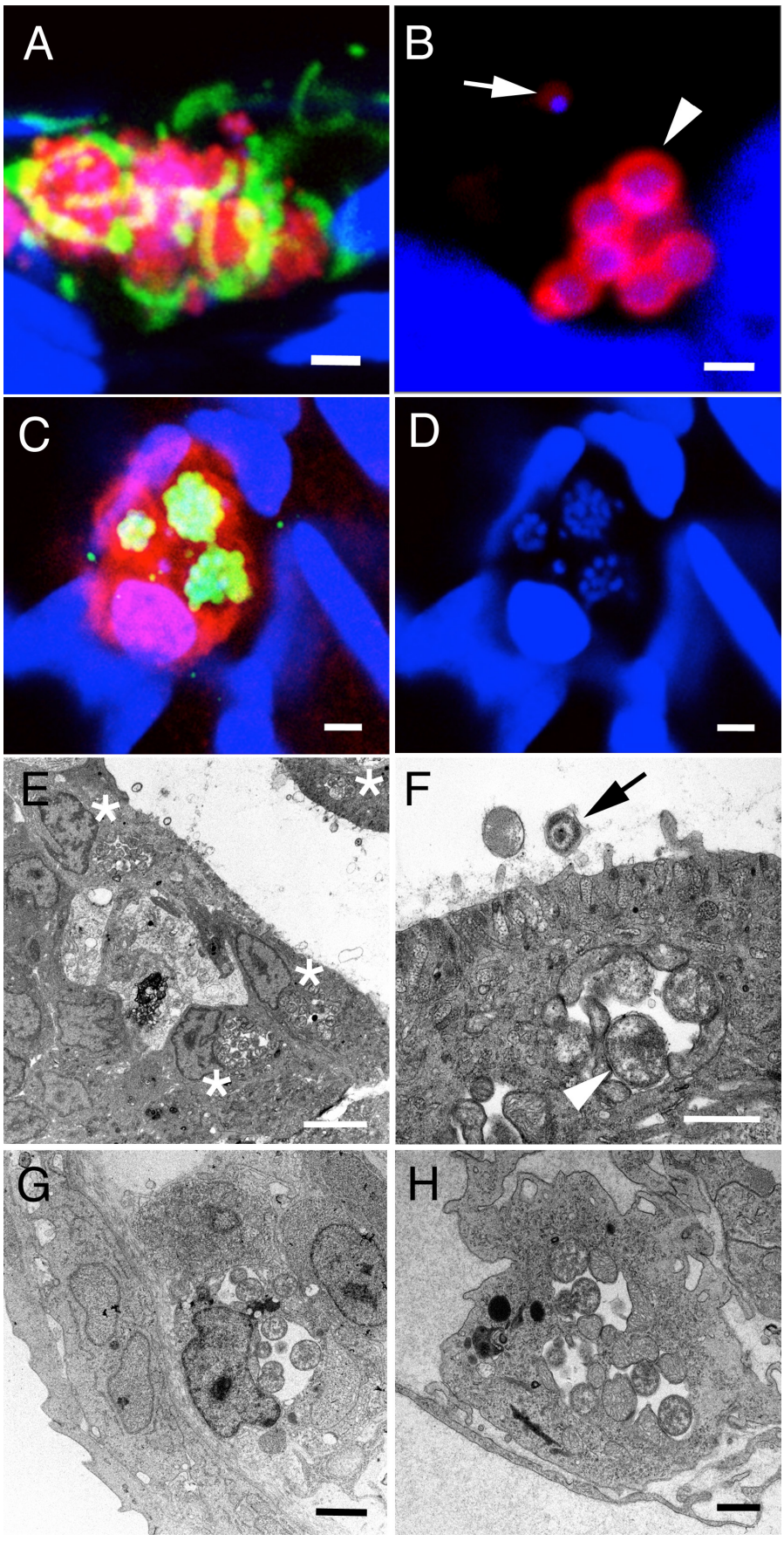
A
B
C
D
E
F
G
H

Figure 3 *Morphology of W. chondrophila inside zebrafish. TEM analysis and CLSM of infected larvae after IF staining with an anti-Waddlia antibody (red in A and B, green in C), an anti-OxPhosIV antibody to stain mitochondria (green in A) and DAPI (blue). W. chondrophila could be found to infect different cells of the zebrafish, predominantly epithelial cells of the swim bladder (A, B, E and F), phagocytes of the innate immune system (C, D and G) and endothelial cells (H). The chlamydial inclusions inside those cells exhibited typical features for Waddlia infection like the transformation from the smaller EBs with condensed DNA (arrow in B and F) to the dividing larger RBs inside the BCV with finely distributed chromatin (arrow head in B and F). Further, host cell mitochondria were readily recruited and closely associated with the inclusion (A), a characteristic for Waddlia infection in all determined host cell types (F, G and H). While epithelial (E) and endothelial cells (H) usually contained a single perinuclear inclusion (asterisks in E), individual phagocytes could harbour several inclusions of dividing bacteria (C, D and G). Scale bars (B,C,D,F,H) 1 µm, (A,G) 2 µm and (E) 5 µm.*

4.4.6 Effect of MyD88 mediated signalling on the infection

To investigate whether MyD88 signalling has a function during *W. chondrophila* infection in zebrafish we knocked down MyD88 expression by injection of a specific morpholino at the 1-cell stage (Bates et al. 2007, Cambier et al. Nature 2014). The resulting knockdown lasted for up to 4 dpf as determined by Western Blot (Fig. 4A). Survival, bacterial load and total phagocyte numbers were compared between control and MyD88 knockdown larvae. During a systemic infection, MyD88 morphant larvae showed a 20% lower survival rate compared to control larvae with a similar initial injection dose of 2 x 10^3 *W. chondrophila* EBs (Fig. 4B). Additionally the bacterial load of MyD88 morphant larvae was slightly higher compared to control larvae at 36 hpi (Fig. 4C). However, differences in macrophage and neutrophil depletion were not significant (Fig. 4D, E).

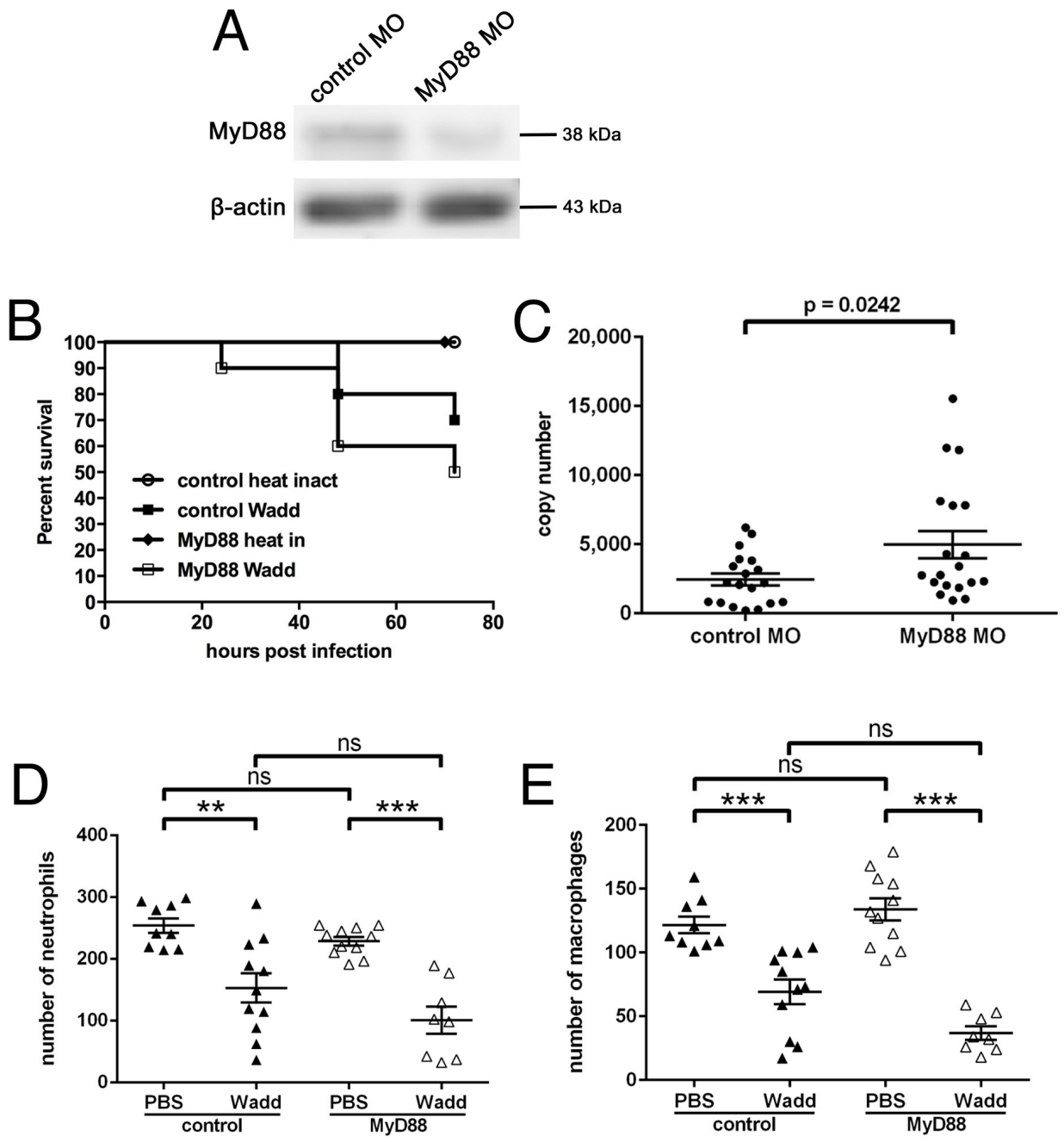

Figure 4 *Morpholino knockdown of MyD88. Western Blot analysis of zebrafish MyD88 at 4 dpf (A) shows a depleted level of the protein in morphant fish. β-actin served as loading control. Survival rates (B) and bacterial load (C) of control and MyD88 MO treated larvae at 36 hpi show differences after intravenous injection of W. chondrophila. Morphant larvae show slightly reduced survival and a higher bacterial load compared to control larvae. Survival rates of 10 larvae for each condition were observed in three independent experiments, respectively. Bacterial load of individual larvae was determined by qPCR targeting the W. chondrophila 16S rRNA sequence Statistical analysis*

*was done by Student's unpaired t-test. * = p < 0.05. Mean values ±SEM are shown by horizontal bars. Total numbers of neutrophils (D) and macrophages (E) of individual Tg(lyzC:dsRed)^{nz50} transgenic and control or MyD88 MO treated larvae at 36 hpi after intravenous injection of PBS or W. chondrophila were counted in Imaris after immunofluorescence staining and CLSM. Immunofluorescence stainings were performed for neutrophils with an anti-dsRed antibody and for macrophages with an anti-Mpeg-1 antibody.. For quantifiacation of phagocytes, CLSM acquired 3D stacks of whole larvae were analysed with the fluorescent spot counting tool in Imaris (Bitplane). The results show a significant depletion of both neutrophils and macrophages in infected larvae, but no differences between MyD88 morphant and control fish could be observed. Statistical analysis was done by one-way ANOVA with Bonferroni's posttest. *** = p < 0.001, ** = p < 0.01, ns = not significant. Mean values ±SEM are shown by horizontal bars*

4.5 Discussion

We present the first zebrafish infection model for an obligate intracellular pathogen, *W. chondrophila*, which produces a non-lethal swim bladder infection and a lethal systemic infection in zebrafish larvae. Primary target cells are epithelial cells of the swim bladder, endothelial cells of the vascular system and phagocytes and granulocytes of the innate immune system. In this study, we show for the first time that *W. chondrophila* can successfully survive and grow within zebrafish macrophages and neutrophils and utilise them as transport shuttles for barrier crossing and dissemination *in vivo*. Moreover by using zebrafish larvae between 2 and 5 dpf our model provides the opportunity to study specifically the reaction of the innate immune system to a *W. chondrophila* infection.

The zebrafish swim bladder is an air-exposed epithelium, regarded as a homologous organ to the mammalian lung, having similar developmental and molecular ontogeny (Flores et al., 2010; Winata et al., 2009). Oral uptake of *W. chondrophila* EBs by 4 dpf larvae results in a swim bladder infection, which can be reproducibly replicated by direct microinjection of *W. chondrophila* into the swim bladder. Since *W. chondrophila* has been linked to cases of pneumonia in humans (Haider et al., 2008), as have related organisms such as *Chlamydia pneumoniae* and *Chlamydia psittaci*, our model could provide a new approach to investigate aspects of human respiratory disease caused by these pathogens *in vivo*.

The injection of live *W. chondrophila* EBs into the swim bladder causes a strong recruitment of neutrophils to the infection site, while neutrophil recruitment is attenuated when using heat-inactivated bacteria. *W. chondrophila* also induces recruitment of macrophages to the swim

bladder, but less intensely. While zebrafish macrophages are known to react to invading bacteria at all locations, zebrafish neutrophils are specialised to phagocytise surface associated bacteria in a vacuum-cleaner-like behaviour (Colucci-Guyon et al., 2011). Thus from our findings, it can be deduced that live *W. chondrophila* EBs adhere more efficiently to the epithelium of the swim bladder compared to heat-inactivated bacteria that probably have an impaired adhesion ability due to denaturation of surface receptors after heat treatment, and that this adhesion results in increased neutrophil recruitment to the infection site.

Intravenous injection of *W. chondrophila* into zebrafish larvae causes a sepsis with strongly impaired blood circulation and subsequent oedema formation, with the ensuing increased mortality correlating with increased dosage. This reaction was only provoked by injection of live infectious EBs and not by the use of heat-inactivated *W. chondrophila*. The infection seems to trigger a strong innate immune response, evidenced by the recruitment and significant depletion of phagocytes. In addition to macrophages, we show for the first time that epithelial cells and neutrophils are among the preferred target cells for infection by *W. chondrophila in vivo*. Infection of endothelial cells has already been shown *in vitro* for *Chlamydia pneumonia* (Gaydos et al., 1996; Godzik et al., 1995), suggesting that these chlamydial pathogens may play a role in the development of vascular disease. Our findings with intravenously injected *W. chondrophila* now provide *in vivo* support for this proposal.

Treatment of the infected larvae with tetracycline significantly reduces the bacterial load and increases the larval survival rate, while treatment with ampicillin is ineffective. Nevertheless, even without antibiotic treatment, the bacterial load starts to decrease after 72 hpi in surviving larvae. The infection is greatly reduced by 4 dpi, although isolated inclusions or infection loci can remain.

Our results show that although both macrophages and neutrophils are susceptible to infection, and can contribute to barrier crossing and further systemic dissemination of *W. chondrophila*, the innate immune system on its own is able to mount an effective counterstrike against *W. chondrophila* infection in surviving larvae. Furthermore we found that MyD88 mediated signalling and hence recognition of *W. chondrophila* by Toll-like receptors contributes to an increased survival and a reduced bacterial load in control larvae compared to MyD88 morphant larvae. These findings indicate a possible role of MyD88 dependent recruitment of innate immune cells like neutrophils for an efficient reaction to counter *W. chondrophila* infection. MyD88 mediated signalling was found to contribute to the generation of an effective early immune response and increased host survival also in a mouse model for *Chlamydia pneumoniae* infection (Naiki et al., 2005). Furthermore, it was shown that *Parachlamydia acanthamoebae* is recognised and internalised by macrophages in a MyD88 independent manner (Roger et al., 2010) while on the other hand in another mouse model

was shown that neutrophil recruitment to *Chlamydia pneumoniae* infection is strongly depending on MyD88 signalling, although it was found as well that recruitment of those neutrophils initially increased the bacterial load (Rodriguez et al., 2005).

Whether MyD88 signalling plays a relevant role at later time points of an infection needs to be further investigated. Possibly a subsequent recognition of the *W. chondrophila* BCV inside infected phagocytes by endosomal Toll-like receptors or cytoplasmic nucleotide binding oligomerization domain (NOD)-like receptors acting in association with MyD88 mediated signalling could lead to a more efficient response of the innate immune system to *W. chondrophila* infection. An essential role for the recognition and defensive reaction induced by NOD-like receptors has already been shown for *Chlamydia pneumoniae* and *Chlamydia trachomatis* (Buchholz and Stephens, 2008; Shimada et al., 2009). Whether *W. chondrophila* is also recognised by intracellular pattern recognition receptors is another key aspect for future studies. The complementary results forthcoming from mouse models for Chlamydia infection and the zebrafish model presented here, emphasises the utility of our approach and offers chlamydial researchers a powerful and readily accessible new tool.

4.6 References

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Chapter 5

Evaluation of zebrafish as a model to study the pathogenesis of the opportunistic pathogen *Cronobacter turicensis*

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Personal contribution:

Planning the experiments, bath-immersion and microinjection experiments, bacterial enumeration by plate counting, statistical analysis, fluorescence light and confocal microscopy, image analysis, live imaging, preparation of the figures, writing of the manuscript

5.1 Abstract

Bacteria belonging to the genus *Cronobacter* (C.) spp. have been recognized as causative agents of life-threatening systemic infections primarily in premature, low-birth weight and/or immune-compromised neonates. Knowledge on the underlying molecular mechanisms of disease development is still scarce. In this study we evaluated the use of the zebrafish model to study the pathogenesis of *C. turicensis* LMG 23827^T, a clinical isolate responsible for two fatal sepsis cases in neonates. Here we show, that microinjection of about 50 CFU into the yolk sac resulted in rapid multiplication of the bacteria and dissemination into the blood stream after 24 hours post infection (hpi), followed by the development of a severe bacteremia and the death of the larvae within 3 days. In contrast, the innate immune response of the embryos was sufficiently developed to control infection after intravenous injection of up to 10^4 CFU of bacteria. Infection studies using a strain devoid of surviving and replicating in human macrophages (*fkpA*) showed that the mutant was highly attenuated in its ability to kill the larvae. In addition, the suitability of the zebrafish model system to study the effectiveness of antibiotics to treat *Cronobacter* infections in zebrafish embryos was exploited in the study. Our data indicate that the zebrafish model represents an excellent vertebrate model to study virulence related aspects of this opportunistic pathogen *in vivo*.

5.2 Introduction

Cronobacter spp. are regarded as opportunistic facultative intracellular pathogens associated with the ingestion of contaminated reconstituted infant formula, that causes serious illness predominantly in low-birth-weight preterm and neonatal infants (Bar-Oz et al., 2001; Hunter and Bean, 2013). Clinical presentation of *Cronobacter* infections include necrotizing enterocolitis (NEC), bacteremia and meningitis, with case fatality rates ranging between 40 and 80 % being reported (Bowen and Braden, 2006; Friedemann, 2009).

The genus *Cronobacter* spp. - as proposed in 2008 - currently consists of seven species according to the „List of prokaryotic names with standing in nomenclature“ (http://www.bacterio.net/allnamesac.html, viewed, 12192014) and encompasses organisms that have previously been identified as *Enterobacter sakazakii* (Iversen et al., 2008; Joseph et al., 2012). Recently, the extension of the genus *Cronobacter* by three more (*Enterobacter*) species was proposed by Brady et al. (Brady et al., 2013) however, re-examination of the biological basis for this suggestion as performed in the study by Stephan et al. does not support further revision of the taxon at this time (Stephan et al., 2011).

Information from epidemiological studies along with *in vitro* mammalian tissue culture assays has shown that *Cronobacter* isolates demonstrate a variable virulence phenotype. So far, only isolates of *C. sakazakii*, *C. malonaticus* and *C. turicensis* have been linked with infantile infections (Joseph and Forsythe, 2011).

Despite the progressive increase in research on *Cronobacter* pathogenesis in the last decade, knowledge on the exact mechanisms of infection is still fragmentary (Jaradat et al., 2014).

As an orally transmitted pathogen *Cronobacter* is thought to gain entrance to the human body through the gastrointestinal tract where it may cause necrotizing enterocolitis (NEC) or, by unknown mechanism(s), may enter the systemic circulation without the manifestation of NEC (van Acker et al., 2001). Once the bacteria have entered the blood stream, they exhibit a tropism towards the central nervous system (CNS) while showing an increased propensity to cause meningitis among low birth weight neonates and infants whereas causing bacteremia or sepsis among slightly higher birth weight infants (Yan et al., 2012). Through crossing of the blood brain barrier (BBB) the pathogen enters the brain, where it causes ventriculitis which could lead to the development of hydrocephalus or forms other sequelae such as cysts or brain abscesses (Bowen and Braden, 2006; Chenu and Cox, 2009).

Most of the data available today have been acquired from *in vitro* studies. *In vivo* studies to confirm and extend these observations from cell culture have largely been concentrated on the neonatal rat, mice or gerbil as model organism (Lee et al., 2011; Mittal et al., 2009; Pagotto and Farber,

2009; Townsend et al., 2007). Although valuable information has been obtained from these studies, the lack of possibilities for a real time analysis and the need for laborious and invasive sample analysis limit the use of mammalian experimental animals.

The nematode *Caenorhabditis elegans* has been alternatively used to study *Cronobacter* virulence factors, exploiting the amenities of the nematode system, such as easy cultivation and transparency (Sivamaruthi et al., 2011). However, invertebrates are genetically not closely related to humans and their immune system shows many differences to the immune system of humans. Hence, other models are needed to address specific questions related to the innate immune response to a specific pathogen in detail.

The zebrafish (*Danio rerio*) may be considered as a hybrid between mouse and invertebrate infection models. The most impressive feature of this model is the possibility to perform non-invasive, high-resolution, long-term time-lapse and time-course experiments to visualize infection dynamics with fluorescent markers in the transparent embryo. Its small size, ease of breeding, high fertility and genetic tractability are further favorable features that make the zebrafish embryo an attractive model organism for science. Furthermore the zebrafish immune system displays many similarities to that of mammals, with counterparts for most of the human immune cell types (Meeker and Trede, 2008). The zebrafish innate immune system starts to develop as early as 24 hours post fertilization (hpf) with primitive macrophages followed by neutrophils at 32-48 hpf. The development of the adaptive immune system lags behind, (Meijer and Spaink, 2011) which gives the opportunity to study independently the innate immunity of the larvae during the first days post fertilization (dpf). This sets zebrafish apart from both *in vitro* and mammalian *in vivo* infection models. Zebrafish larvae have already been used to study infections of other bacterial pathogens, (Kanther and Rawls, 2010) including *Listeria monocytogenes*, *Salmonella typhimurium* and *Shigella flexneri* (Levraud et al., 2009; Mostowy et al., 2013; van der Sar et al., 2003).

In this study, we exploited the advantages of the zebrafish to investigate infections by *Cronobacter turicensis* LMG 23827^T *in vivo*. We show here that *Cronobacter* causes lethal infection in zebrafish larvae sharing similarities with human cases. After having successfully established the experimental parameters, the model was evaluated using a strain devoid of expressing a gene, which has recently been described as virulence factor in *C. turicensis*. In addition, the model was used to study the effectiveness of different antibiotics to treat *Cronobacter* infection in zebrafish larvae.

5.3 Materials and Methods

5.3.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *C. turicensis* LMG 23827^T , a clinical isolate responsible for the death of two neonates in Zurich 2006 has been object of research before (Carranza et al., 2010; Carranza et al., 2009; Essers et al., 2006; Stephan et al., 2011). Construction of the *C. turicensis* LMG 23827^T Δ*fkpA* mutant, the complemented mutant *C. turicensis* LMG 23827^T Δ*fkpA*::*fkpA*Tet^R as well as the mutant carrying the complementation vector pCCR9Tet^R only (*C. turicensis* LMG 23827^T Δ*fkpA*::pCCR9Tet^R) is described in detail in the study by Eshwar et al. (2015, submitted).

The green fluorescent protein (GFP) expressing strain *C. turicensis* LMG 23827^T::GFPKan^R was constructed during the study by Schmid et al., 2006. For selection purposes during zebrafish embryo infection experiments *C. turicensis* LMG 23827^T::dsREDTet^R, *C. turicensis* LMG 23827^T Δ*fkpA*::dsREDTet^R as well as *E. coli* DH5a::dsREDTet^R were constructed in this study by transformation of vector pRZT3::dsREDTet^R using standard methods. Plasmid pRZT3::dsREDTet^R was a kind gift from by A. M. van der Sar (Medische Microbiologie, Vrije Universiteit medisch centrum, Netherlands). For cultivation strains were grown in 10 ml Luria–Bertani (LB) broth, over night at 37°C with gentle shaking. *C. turicensis* LMG 23827^T variants/mutants were cultivated in LB broth supplemented where appropriate with either tetracycline at 50 mg/l or kanamycin at 50 mg/l.

For microinjection experiments bacteria were harvested by centrifugation at 5000 x g for 10 min and washed once in 10 ml DPBS. After a second centrifugation step the cells were resuspended in DPBS and appropriate dilutions were prepared in DPBS.

5.3.2 Zebrafish lines and husbandry

Zebrafish (*Danio rerio*) strains used in this study were predominantly *albino* lines as well as transgenic fish of the *Tg(lyz:DsRED2)nz50* line that produce red fluorescent protein (RFP) in neutrophils, received as a kind gift from Professor Philip Crosier, University of Auckland (New Zealand) (Hall et al., 2007). Adult fish were kept at a 14/10 hours light/dark cycle at a pH of 7.5 and 27°C. Eggs were obtained from natural spawning between adult fish which were set up pairwise in separate breeding tanks. Embryos were raised in petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) supplemented with 0.3 μg/ml of methylene blue at 28°C. From 24 hpf, 0.003 % 1-phenyl-2-thiourea (PTU) was added to prevent melanin synthesis. As *albino* lines lack melanised chromophores, no PTU treatment was performed on these. Staging of embryos was performed according to Kimmel et al. (Kimmel et al., 1995).

Research was conducted with approval (no. 216/2012) from the Veterinary Office, Public Health Department, Canton of Zurich (Switzerland).

5.3.3 Microinjection experiments

Injections were conducted using borosilicate glass microcapillary injection needles (Science Products, 1210332, 1 mm O.D. x 0.78 mm I.D.) and a PV830 Pneumatic PicoPump (World Precision Instruments). Prior to injections embryos of 2 dpf were manually dechorionated and anesthetised with 200 mg/l buffered tricaine (MS-222). Afterwards embryos were aligned on an agar plate and injected with 50 to 10^4 CFU in 1-2 nl volume of a bacterial suspension in DPBS either directly into the blood circulation, the hindbrain ventricle or into the yolk sac. The volume of the injected suspension was previously adjusted by injection of a droplet into mineral oil and measurement of its approx. diameter over a scale bar. In initial experiments we determined the inoculum size by direct microinjection onto agar plates. However, we found that plating 5 embryos individually (see below) immediately after microinjection (0 hpi) resulted in more accurate determination of the numbers of CFU actually injected.

After injections infected embryos were allowed to recover in a petri dish with fresh E3 medium for 15 min. To follow infection kinetics, embryos were transferred in 6-well plates in groups of about 15 embryos in 4 ml E3 medium per well, incubated at 28°C and observed for signs of disease and survival under a stereomicroscope twice a day.

Five embryos or larvae were collected at each time point, generally 0, 15, 24, and 48 hpi, and individually treated for bacterial enumeration. Sampled larvae were euthanized with an overdose of 4 g/l buffered tricaine and transferred into different buffers and fixatives for subsequent analyses respectively.

5.3.4 Bacterial enumeration by plate counting

The larvae were transferred to a 1.5-ml Eppendorf tube and disintegrated by repeated pipetting and vortexing for 3 minutes in 1 ml PBS supplemented with 1% Triton X- 100. Subsequently, 100 ml of this mixture was plated onto LB selective plates (i.e. tetraclycline 50 mg l^{-1} for strains harbouring pCCR9 or pRZT3::dsRED or kanamycin 50 mg/l for selection for *C. turicensis* LMG 23827^T::GFP). Plates were incubated up to 48 h at 37 °C.

For survival assays, embryos were similarly microinjected and maintained individually in 24-well plates in E3 medium at 28°C. At regular time points after infection, the number of dead larvae was determined visually based on the absence of a heartbeat.

5.3.5 Drug testing

For drug screening infected embryos were transferred after yolk injections into 24-well plates with one embryo in each well in 1 ml E3 containing either 8 mg/l ampicillin, 8 mg/l tetracycline, 4 mg/l nalidixic acid or were left untreated (no drug added). Drugs were added to the water at their respective concentrations. Samples were taken and analyzed like described above.

5.3.6 Statistical analysis

Statistics and graph design were done with GraphPad Prism 6 (GraphPad Software, United States). Experiments were performed at least three times, unless stated otherwise. Numbers of CFU of groups of individual larva at various time points and under various conditions were tested for significant differences by one-way ANOVA with Bonferroni's posttest. ***, $p<0.001$; ns, not significant.

5.3.7 Light-Microscopy, fluorescence imaging and image analysis

For histological examination whole zebrafish larvae were fixed in 4% paraformaldehyde at 4°C and embedded in cubes of cooked egg white in order to position them correctly for histological sections. These cubes containing the larvae were dehydrated in an alcohol series of ascending concentrations ending in xylene and afterwards embedded in paraffin. Paraffin blocks were cut in 2-3 μm thin sections, mounted on glass slides and stained using a routine protocol with haematoxylin and eosin (HE).

Overview images were done with an upright light microscope (Olympus BX61) with both bright field and fluorescence modules. The fluorescence filter cube used was optimized for DAPI/FITC/TRIC. For higher resolution images, 3D-image stacks of whole mount samples were prepared using a confocal laser scanning microscope (CLSM, Leica TCS SP5, Leica Microsystems). GFP, dsRED and DAPI were sequentially excited with the 405 nm, 488 nm and 561 nm laser lines respectively, with emission signals collected within the respective range of wave lengths. 3D image stacks were collected sequentially (to prevent blue-green–red channel cross-talk) according to Nyquist criteria and deconvolved using HuygensPro via the Huygens Remote Manager v2.1.2 (SVI, Netherlands). Images were further analysed with Imaris 7.6.1 (Bitplane, Zurich, Switzerland) and aligned with Adobe Photoshop Elements 12.

5.3.8 Transmission Electron Microscopy

For electron microscopy, larvae were fixed in a mixed solution of 1 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.5 at 4°C overnight. Afterwards samples

were prepared for embedding into epoxy resin and for transmission electron microscopy according to standard procedures. Epoxy resin blocks were screened for larvae by using semithin sections (1 μm) which were stained with toluidine blue (Sigma-Aldrich) to visualize tissue. Ultrathin sections (80 nm) were mounted on copper grids (Merck Eurolab AG, Dietlikon, Switzerland), contrasted with uranyl acetate dihydrate (Sigma-Aldrich) and lead citrate (Merck Eurolab AG) and investigated using a Philips CM10 transmission electron microscope. Images were processed with Imaris (Bitplane) and assembled for publication using Adobe Photoshop.

5.3.9 Multidimensional digital imaging microscopy of zebrafish embryos

Living zebrafish embryos were examined with a ZEISS Axiovert 200 MarianasTM inverted microscope, equipped with a motorised stage (stepper-motor z-axis increments: 0.1 mm), and both a Cy- 3 as well as a DIC cube. A cooled CCD camera [Cooke Sensicam (Cooke, Tonawanda, NY), 1280 ¥ 1024 pixels] recorded images with true 16-bit capability. The camera is linear over its full dynamic range (up to intensities of over 4000) while dark/back- ground currents are typically <100. Under the present set of chosen dyes and loading conditions, exposures ranged from 2 ms to 2 s. This ensures that genuine signals are at least >5 ¥ higher than autofluorescence (typically about 200–500 in these embryo's). Exposures, objective, montage and pixel bin- ning were automatically recorded with each image stored in memory. The microscope, camera and all other aspects of data acquisition as well as data processing were controlled by SlideBookTM software [SlideBook version 3.1 (Intelligent Imaging Innovations, Denver, CO)]. All live microscopy was performed with a custom 10 ¥ or 40 ¥ air lens (ZEISS). The data acquisition protocol included both time lapses and series of confocal optical planes to obtain 3D definition.

5.3.10 Confocal Live Imaging

To perform high resolution confocal live imaging, injected larvae were positioned in 35 mm glass-bottom dishes (Inagaki- Iwaki). A 1 % low-melting-point agarose solution was used to cover the entire larva to immobilize the larva in the dish. The immobilized larvae were then covered with 2 ml fish water containing Tricaine (Sigma). Confocal microscopy was performed at 26 °C. A Leica SP8 automated upright confocal laser scanning microscope allowing simultaneous acquisition of 3 fluorescent channels and 1 transmitted light channel (BF or DIC) was used. The detection system in this microscope is equipped with two PMT and a Hybrid detector (HyD) and a 20x water immersion objective (HC PL APO NA-0.5 WD-3.5 mm) was used to image live infected larvae. The 4D files produced by the time-lapse acquisitions were processed, clipped, examined and interpreted using the Imaris software (Bitplane). Acquired Z-stacks were projected using maximum intensity projection and exported as AVI files. Photoshop software was used to handle frames

captured from the AVI files to mount figures. AVI files were also cropped and annotated with Imaris software, then compressed and converted into QuickTime movies with the QuickTime Pro software.

5.4 Results

5.4.1 Infection of zebrafish larvae with *Cronobacter* via microinjection is lethal

In an effort to develop a *Cronobacter turicensis* infection model in a genetically tractable vertebrate model host, we investigated whether strain *C. turicensis* LMG 23827^T, a strain originally isolated from a fatal neonatal infection could lethally infect zebrafish embryos at 2 dpf. Initial experiments to infect zebrafish with this strain by immersing dechorionated embryos in a suspension of strain LMG 23827^T failed. Lethality required high concentrations of strain LMG 23827^T or LMG 23827^T::GFP (10^9 – 10^{10} CFU ml^{-1}) and experiments were not reproducible in our hands (data not shown). Similar results were reported for co-infection experiments using other pathogens under static immersion conditions (van Soest et al., 2011). Moreover, using the bath immersion experimental set up we were unable to establish a stable infection in the digestive system of the larvae. Similar observations were reported in the study by Levraud et al., showing that zebrafish embryos were not susceptible to oral infection with *Listeria monocytogenes* (Levraud et al., 2009).

Therefore we next focused on the possibility to introduce LMG 23827^T or LMG 23827^T::GFP into 2 dpf old embryos directly by microinjection into the yolk sac, the common cardinal vein or the hindbrain ventricle, thereby exploiting the advantage of easy fluorescent tracking by using the GFP transgenic strain *C. turicensis* LMG 23827^T::GFP with concentrations ranging from 50 to 10^4 CFU. Yolk injections were performed into the posterior part of the yolk sac prior to the extension part to prevent perforation of the common cardinal vein, which widely covers the anterior part of the yolk. After injections embryos were transferred into 24-well plates containing fresh E3 medium and further incubated at 28°C. Signs of disease, larval survival, fluorescence pattern of bacteria, and bacterial load were examined over time. The bacterial load was determined by counting CFU of homogenates of whole individual larva plated on agar plates containing kanamycin for selection purposes at 37°C over night.

Intravenous injections of up to 10^4 CFU and hindbrain injections of up to 10^3 CFU did not result in an apparent infection. Bacteria were cleared from the system within the first 24 hpi and larvae showed no indication of disease or mortality (data not shown). However, injections of as little as 50 CFU into the yolk resulted in rapid replication of *Cronobacter* inside the yolk sac of about two log units within 24 hpi and a subsequent spreading into the larval blood stream between 24 and 48 hpi (Figure 1A). The bacterial load continued to increase for another log unit at the same time (Figure

1E). Confocal imaging revealed bacteria accumulating at the surface of the yolk sac before further spreading into the larvae (Figure 1B). Bacteria inside the yolk could be found dividing by binary fission (Figure 1C and D). Mortality after yolk injections raised up to 100 % at 72 hpi, while microinjection of equal or greater numbers of *E. coli* DH5a::dsRED resulted in complete survival of infected larvae (Figure 1F).

The traverse of *Cronobacter* to the bloodstream could be observed throughout the whole length of the boundary between yolk and vasculature (Figure 2A-D), followed by an accumulation of bacterial clusters in the capillaries of the trunk and the eyes (Figure 2E and F).

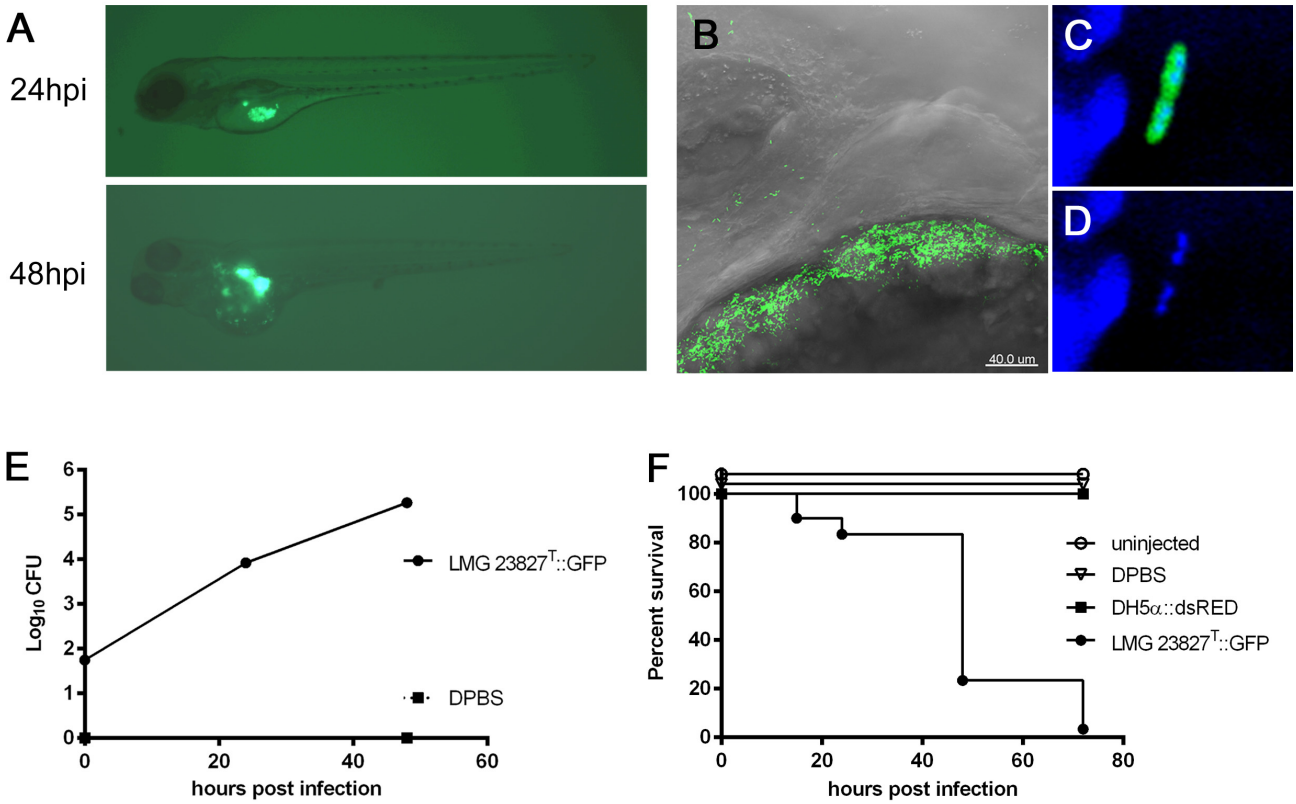

Figure 1 *Injection of C. turicensis into the yolk of 2 dpf embryos causes lethal infection. (A) Appearance of larvae at 24 and 48 hpi under a fluorescence light microscope after injection of 50 CFU of C. turicensis LMG 23827^T::GFP into the yolk sac. At 24 hpi, replication of GFP expressing bacteria and further spreading into the yolk are visible. At 48 hpi, continuous replication and spreading inside the whole yolk sac and also into further tissues of the larvae can be observed. (B) CLSM acquired 3D stack showing a part of the border between the yolk sac and the larva in a region close to the head. GFP expressing bacteria are accumulating on the surface of the yolk. Some have already crossed the barrier, distributing in the larva. (C and D) Inside the yolk, many dividing bacteria can be observed, confirmed by DAPI staining of bacterial and host DNA. The*

images show merged channels for DIC/GFP (B), DAPI/GFP (C) or DAPI alone (D). (E) Mean growth curve of C. turicensis inside infected larvae with a starting inoculum of about 50 CFU. Enumeration was done by plating homogenates of whole indiviual larvae at different time points on selective agar plates at 37°C and subsequent counting of bacterial colonies. (F) Survival rates of larvae injected with 50 CFU C. turicensis LMG 23827^T::GFP or 50 CFU E. coli DH5α or 1 nl DPBS or left uninjected, following incubation at 28°C for 72 hpi.

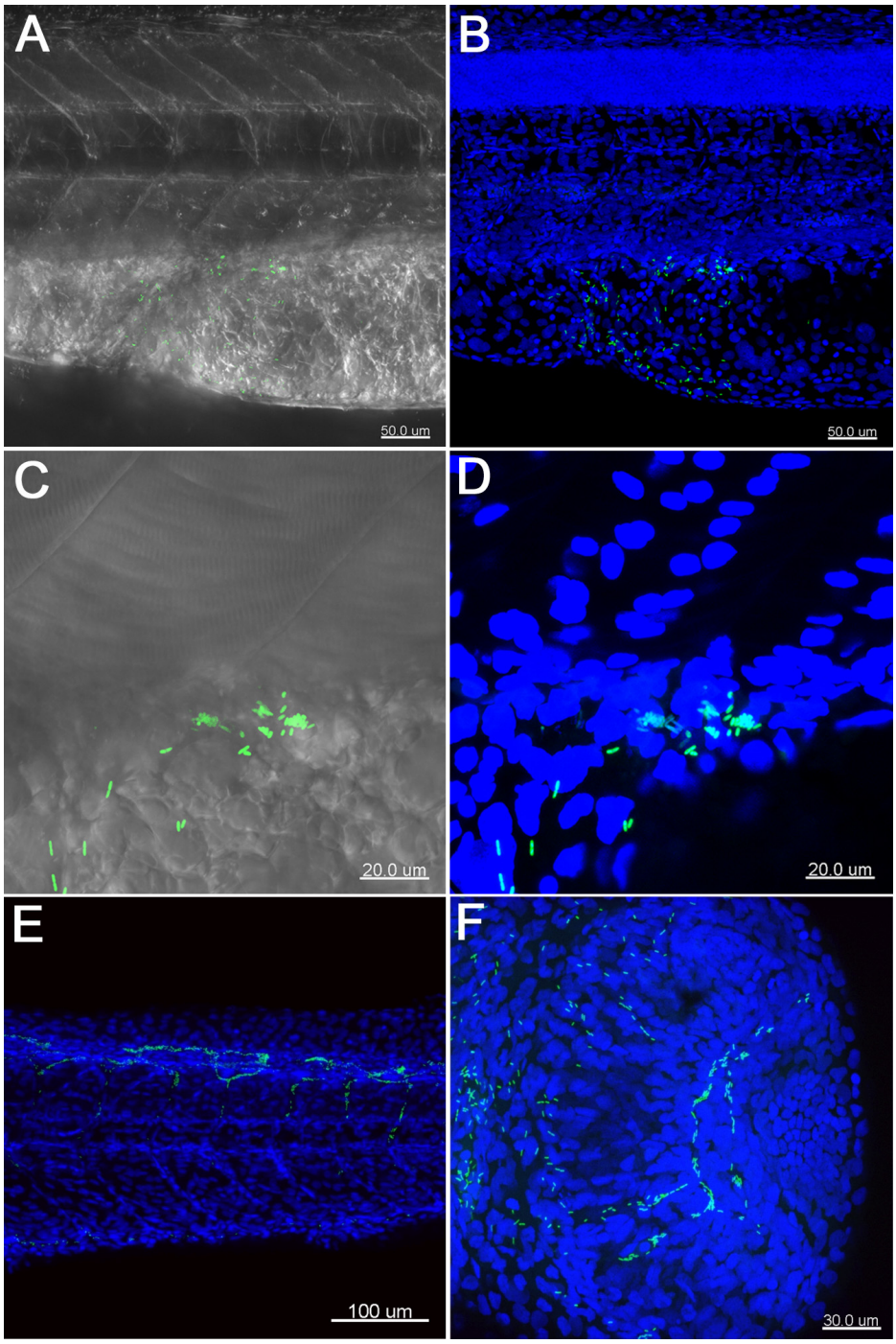

Figure 2 *Confocal imaging of DAPI stained larva after yolk injection of GFP expressing Cronobacter reveals bacteria inside the yolk and the bloodstream of the larva. At 24 hpi, the bacteria have spread from the initial injection site into the extension part of the yolk sac (A and B). The rod-shaped bacteria are forming clusters and replicate by binary fission near the barrier between yolk and the vasculature of the larva (C and D). At 48 hpi, numerous bacteria are visible in the blood circulation of the trunk (E) and the eye (F), forming clusters and accumulating in the capillaries. DIC/GFP (A and C) and DAPI/GFP (B, D, E and F) channels are merged respectively.*

5.4.2 Progression and pathology of the infection

Larvae that were injected into the yolk sac showed at 30-48 hpi, small 1 x 2 μm, rod shaped bacteria intracytoplasmatically in macrophages and immature neutrophils in the yolk sac (Figure 3A and B) and in the lumina of several blood vessels especially close to the eyes and brain (Figure 3C and D). The number of macrophages and neutrophils within the yolk sac lining the wall was increased compared to control animals. Within the blood vessels the number of leukocytes were increased (leukocytostasis) which could be a sign for sepsis. Many leukocytes showed degeneration as karyorhexis, karyopyknosis and hypereosinophilia of their cytoplasm. The presence of neutrophils was confirmed by confocal imaging where labelled neutrophils were associated with the bacteria inside the yolk sac or blood vessels (Figure 4).

TEM images showed long rods of 2 μm length and 1 μm width that were laying free in the protein of the yolk sac or intracytoplasmatically in macrophages (Figure 3E). The bacteria contained a thin cell wall, typically for Gram-negative rods and a loose chromatin pattern. Some bacteria were dividing which could also been shown by confocal microscopy. Some leukocytes containing bacteria showed degeneration as large pale intracytoplasmatic vacuolation, crystolysis and swelling of mitochondria, increase of lipid globules or dilation or even fragmentation of Golgi or ER (Figure 3F).

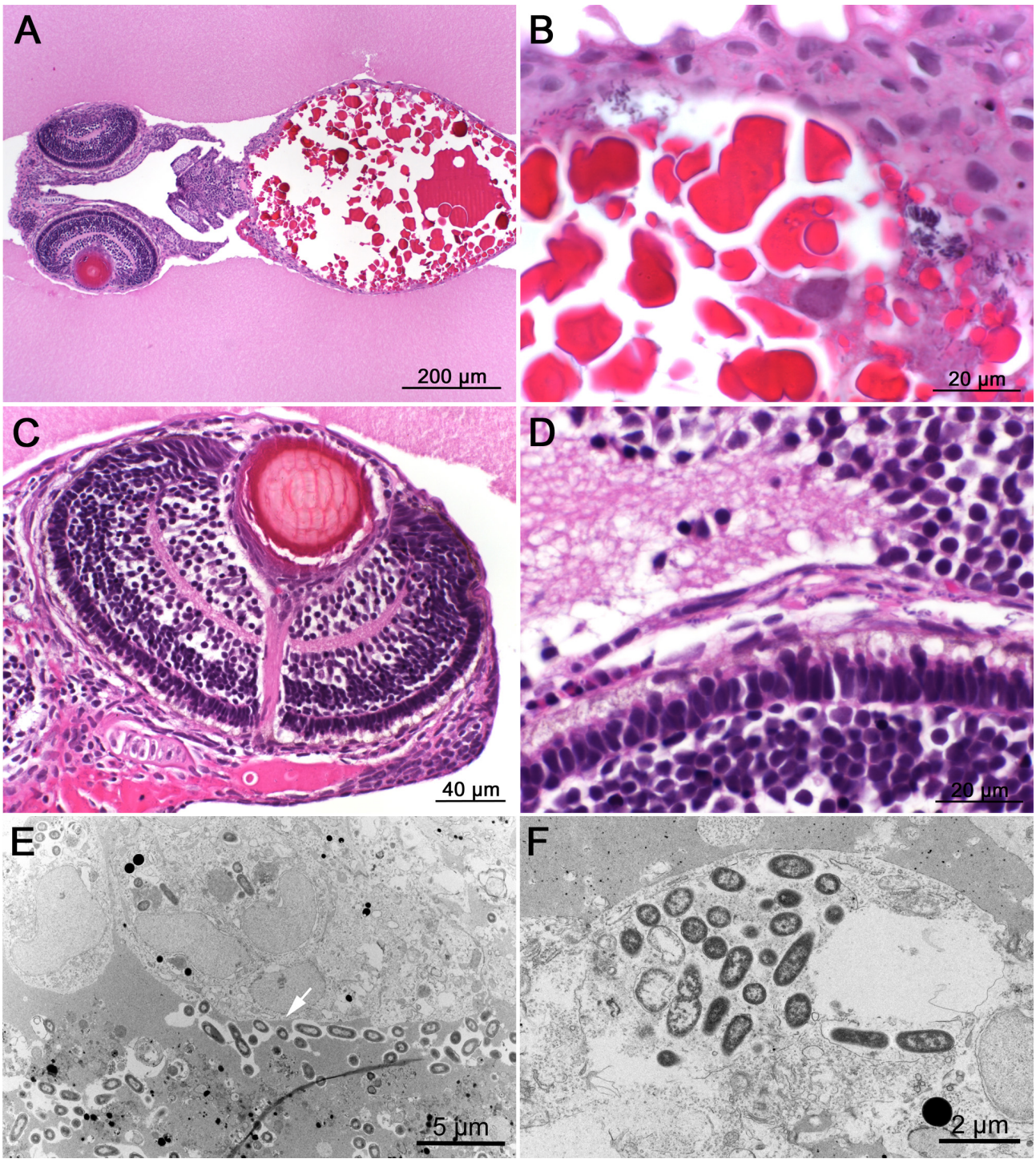

Figure 3 *Pathology of the infection. Histological HE-stained sections of infected larvae at 48 hpi. (A) The overview shows a coronary section through the yolk and the head region of a whole larva embedded in egg white. (B) Under higher magnification clusters and single bacteria are visible inside the yolk (black arrows). Innate immune cells like macrophages and neutrophils can be observed being recruited to the yolk sac and taking up these bacteria. (C and D) Further bacteria can be observed in the blood circulation, accumulating in capillaries of the eyes and brain (black arrows), followed by recruitment of innate immune cells. (E) TEM imaging of the barrier region*

between yolk and larva shows bacteria distributed inside but also lining up at the border of the yolk (white arrow). (F) Some bacteria are phagocytized by macrophages and some can be found in the cytoplasm of different cells lining the yolk barrier. Some of these cells are degrading, recognized by swelling and fragmentation of their organelles.

5.4.3 Innate immune response to *Cronobacter* infection (CLSM Leica SP8 – live imaging)

To visualize the dynamics of *Cronobacter* replication and the innate immune reaction to the infection inside the yolk, we utilized transgenic 2dpf zebrafish embryos of the *Tg(lyz:DsRED2)nz50* line, which are harboring RFP expressing neutrophils. Those embryos were injected with a dose of about 50 CFU of GFP expressing *C. turicensis* LMG 23827^{T}::GFPKan^{R} into the yolk sac at 2 dpf. The *Cronobacter*-host interactions were captured in real-time at 24 hpi using high-resolution confocal laser scanning microscopy for a time course of about 2 hours. We observed rapid replication of *Cronobacter* inside the yolk forming several clusters of motile bacteria. Over time an increasing number of red fluorescent neutrophils was recruited to the yolk taking up the bacteria but were being unable to control proliferation and spreading of *Cronobacter* (Figure 4).

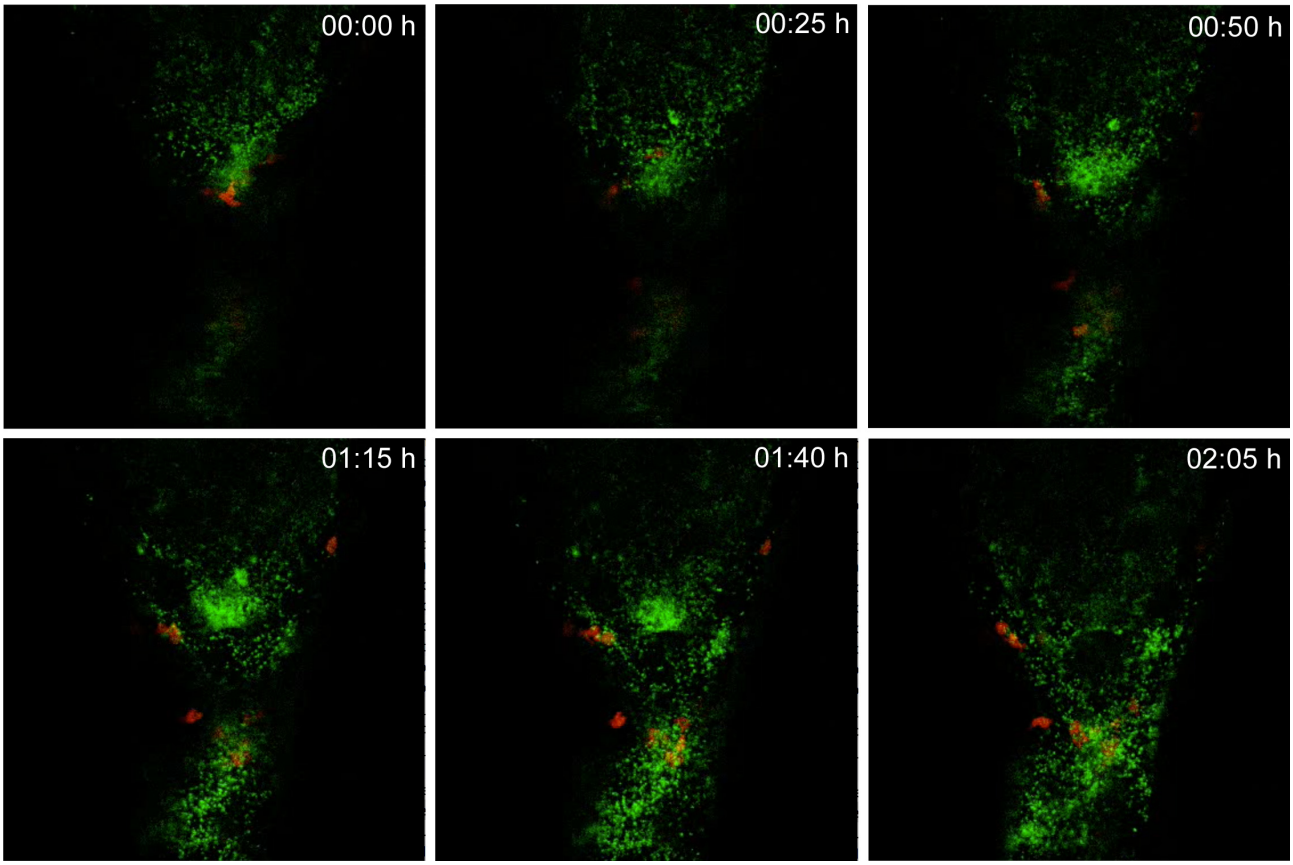

Figure 4 *C. turicensis rapidly replicates inside the yolk and initiates an innate immune response with recruitment of neutrophils. Live imaging of the replication of C. turicensis LMG 23827^T::GFP after injection of 50 CFU into the yolk of 2 dpf transgenic Tg(lyz:DsRED2)nz50 zebrafish embryos, possessing red fluorescent neutrophils. Rapid replication and clustering of the bacteria can be observed, which readily induced recruitment of neutrophils into the yolk sac.. Live imaging was performed using a CLSM Leica SP8 over a time course of about 2 hours (h).*

5.4.4 Drug screening

In a next step we wanted to determine whether the zebrafish model may be suitable for testing the effectiveness of antimicrobial agents to clear infections with *C. turicensis* LMG 23827^T. Prior to these experiments, the minimum inhibitory concentrations (MICs) of a selection of antimicrobial drugs belonging to different antibiotic classes were determined for the wild type *C. turicensis* LMG 23827^T as well as the GFP expressing strain *C. turicensis* LMG 23827^T::GFPKan^R using E-test strips (bioMerieux, Marcy-l-Etoile, France) on Müller Hinton agar, according to the recommendations of the manufacturer. Following MIC values (in mg/l) levels were determined for both strains: ampicillin: 0.75, tetracycline 1.5, cephalothin: 6, rifampicin: 2, gentamycin: 0.38, polymyxin B: 0.094, nalidixic acid: 0.5 and chloramphenicol: 64. Conversion of the MIC data into qualitative categories using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints suggested that *C. turicensis* LMG 23827^T was susceptible to all tested antibiotics with exception of rifampicin and chloramphenicol. There was no variation among the wild type and its GFP variant (data not shown).

Based on these findings we decided to test the activity of ampicillin, tetracycline and nalidixic acid against *Cronobacter in vivo* in the zebrafish infection model. Since all three antibiotics are water-soluble they were administered to the fish water after yolk injections of *Cronobacter* or DPBS as control. Another control group was infected but left without any treatment. The survival rate (Figure 5B) and the bacterial load (Figure 5C) were determined for individual larvae by microscopic observation and plate count enumeration. The distribution of *Cronobacter* within infected larvae was followed by fluorescence microscopy after the injection of the GFP expressing strain (Figure 5A). While treatment with tetracycline had no significant effect on the bacterial load and survival, treatment with ampicillin significantly reduced the bacterial load compared to untreated larvae, but did not promote an increased survival. However, treatment with nalidixic acid had a significant impact on both bacterial load and survival of infected larvae. At 24 hpi *Cronobacter* could be no more detected in these larvae neither by plate count nor by fluorescence microscopy. Furthermore

the survival rate was close to 100 % at 72 hpi. Interestingly, during treatment with nalidixic acid, the formation of pericardial edema was observed in nearly 100 % of all larvae.

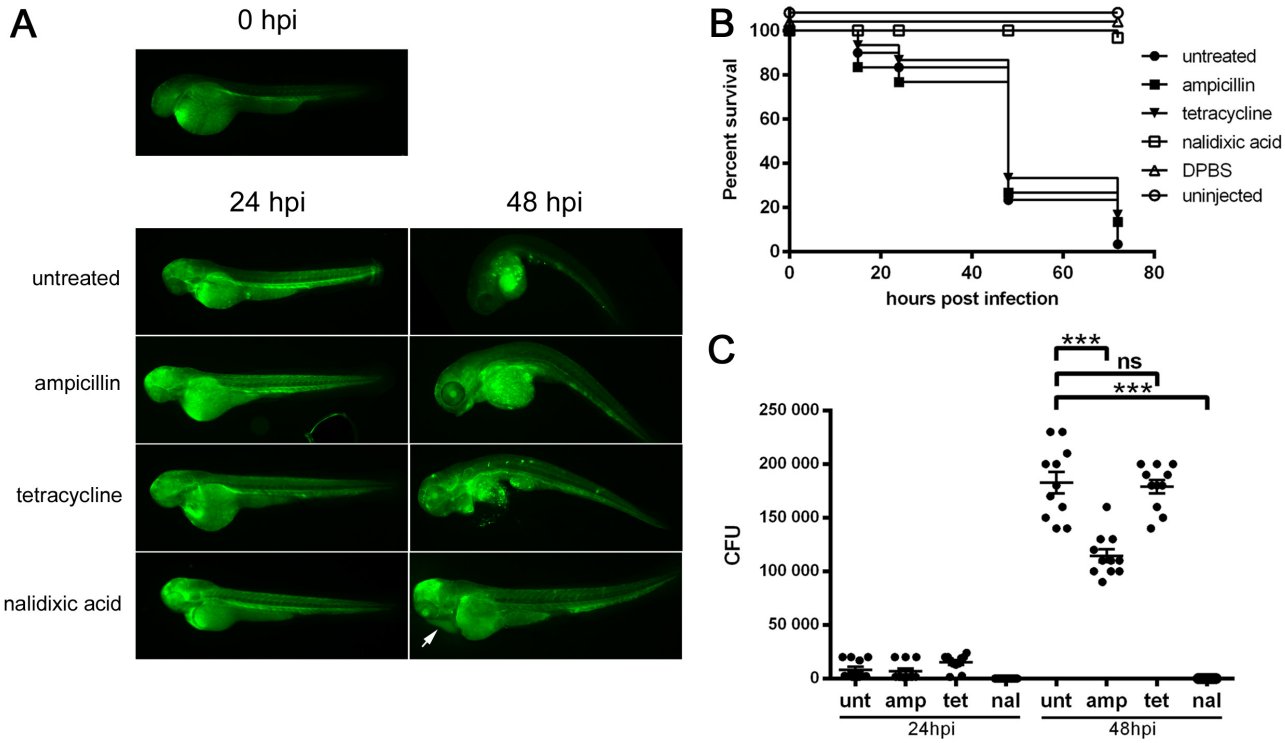

Figure 5 *Effect of antibiotic drugs on an infection of C. turicensis in zebrafish larvae. (A) Fluorescence light microscope appearance of representative larvae at different time points after the injection of C. turicensis LMG 23827^T::GFP into the yolk sac at 2 dpf and subsequent treatment with 8 μg/ml ampicillin or 8 μg/ml tetracycline or 4 μg/ml nalidixic acid or without treatment. Larvae treated with nalidixic acid exhibited formation of pericardial edema at 48 hpi (white arrow) (B) Survival rates of infected larvae with and without treatment with various antibiotics and of control larvae injected with DPBS or uninjected controls are shown. (C) Quantification of the bacterial load of indivdual larvae at different time points and under different treatment conditions are shown. Significant differences could be observed at 48 hpi for the treatment with ampicillin and nalidixic acid compared to untreated larvae. Statistical analysis was done by one-way ANOVA with Bonferroni's posttest. ***, p<0.001; ns, not significant. Mean values ±SEM are shown by horizontal bars.*

5.4.5 FkpA is an important virulence factor for *Cronobacter turicensis* infection in zebrafish embryos

Given the previous results and observations concerning the behavior of *Cronobacter* and innate immune cells after injection into the yolk sac, we concluded that internalization and survival of

Cronobacter cells in professional phagocytes of the innate immune system like macrophages present in the yolk and/or the blood stream, plays a key role during the infection process. In a recent study the eminent role of a functional FkpA (also known as macrophage infectivity potentiator (MIP) like protein) in survival and replication in human macrophages was reported for *C. turicensis* LMG 23827^T (Eshwar et al., 2015, submitted). We decided to test a *ΔfkpA* in frame mutant for attenuated pathogenicity in infection experiments using the above described experimental set-up. *C. turicensis* LMG 23827^T::dsRED served as control. The bacterial load in *ΔfkpA*::dsRED mutant (*C. turicensis* LMG 23827^T Δ*fkpA* ::dsRED) injected larvae was significantly lower compared to the control group (Figure 6B and C). Furthermore the survival rate at 48 hpi raised to about 70 % for *ΔfkpA*::dsRED mutant injected larvae (Figure 6A), thus confirming the role of this gene as a pathogenicity factor also during *Cronobacter* infection in zebrafish embryos. Injection experiments using the complemented mutant strain (*C. turicensis* LMG 23827^T Δ*fkpA::fkpA*) resulted in a lower survival rate and a higher bacterial load whereas control experiments using the mutant strain transformed with the vector alone (*C. turicensis* LMG 23827^T Δ*fkpA*::pCCR9) yielded survival rates and bacterial loads comparable to the ones observed in the *ΔfkpA*::dsRED mutant injection experiments (Figure 6A-C).

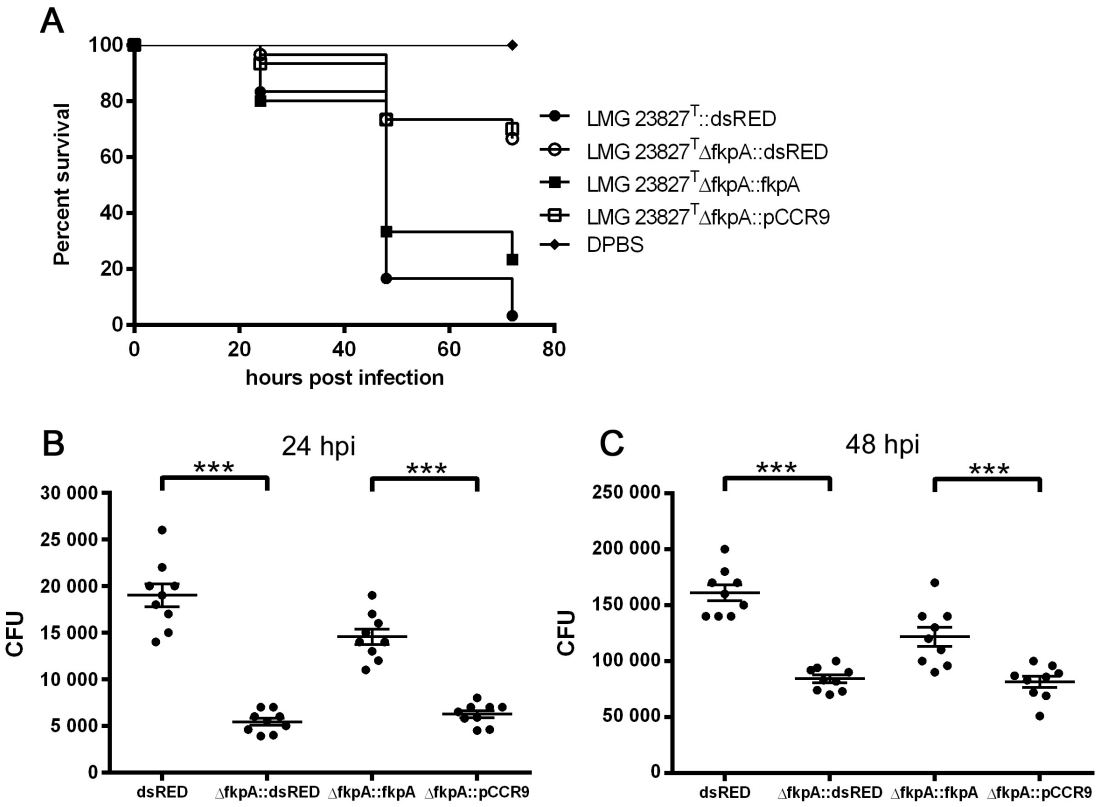

Figure 6 *The mutant C. turicensis LMG 23827^T ΔfkpA shows attenuated pathogenicity in zebrafish larvae. (A) Survival rates of larvae injected with 50 cfu of C. turicensis LMG 23827^T::dsRED or*

*LMG 23827^T ΔfkpA ::dsRED or LMG 23827^T ΔfkpA::fkpA or LMG 23827^T ΔfkpA::pCCR9 or 1 nl of DPBS alone as negative control at 2 dpf into the yolk are shown. (B and C) Quantification of the bacterial load of individual larvae at 24 and 48 hpi shows significant differences between wildtype and mutant bacteria. Statistical analysis was done by one-way ANOVA with Bonferroni's posttest. ***, p<0.001. Mean values ±SEM are shown by horizontal bars.*

5.5 Discussion

In an effort to adopt the zebrafish model to study these human opportunistic pathogens several set-ups were examined throughout the study. As infecting the embryos via the oral route proved unsuccessful, we focused on introducing the pathogens into the animals circulation system via microinjection. Since it was shown that *Cronobacter* spp. are unable to replicate inside blood or serum, one could speculate, that this fact enables the innate immune system to cope with such high CFU levels injected into the embryo's blood circulation. In fact, it has been reported that *Cronobacter* spp. exhibit a "moderate" capability to survive in human blood or serum. Thus it was shown for *C. sakazakii,* which is closely related to *C. turicensis*, that a minimum of two log reduction of a bacterial inoculum took place within 30 min exposure in 50 % human blood (Schwizer et al., 2013). However, injection of as high as 10^4 CFU into 2 dpf old zebrafish larvae did not result in clinical signs of infection; bacteria were quickly cleared by the embryonic innate immune system within 24 hpi.

Therefore we exploited the yolk as potential infection site. We could show that following injection of as few as 50 CFU, bacteria were rapidly replicating both freely in the yolk as well as internalized in primitive macrophages and neutrophils present in the yolk sac. The affected leucocytes were neither killed rapidly, nor did the phagocytosed bacteria escape from these cells. At 24 hpi free bacteria as well as infected macrophages were lining up near the border of the yolk sac from where a traverse into the blood vessel and the surrounding tissue was observed. At later time points (30hpi – 48hpi) bacteria colonized the lumina of small blood vessels especially close to the eyes and brain. Accumulation of bacteria in the capillaries of the eyes are typical features for septicemia and bacteremia in fish. At these sites, the bacteria were seen to form micro-colonies in which they replicated and from which they were shed into the circulation. Thus, during infection in zebrafish embryos a combination of extracellular and intracellular replication of *Cronobacter* could be observed.

In a next experiment a selection of antimicrobial compounds was tested for their capability to cure a *Cronobacter* infection in the embryos by application of the drugs to the fish water. Given the fact that for all antibiotics concentrations were applied, which were previously shown to be lethal for the

bacteria *in vitro*, we can only speculate why treatment with ampicillin and tetracycline was not effective in curing the infections *in vivo*. One explanation may be that the molecules are not reaching lethal concentrations at the predominant infection site inside the yolk, which could be influenced by the size of the molecules, the local pH or the acid/base character of the antimicrobial substance. Nalidixic acid was the only drug, which proved efficient in killing the bacteria inside the host. Although survival rate of infected fishes were close to 100 % after 72 hpi, pericardial edema was observed during the treatment with the drug. Interestingly, similar adverse effects, namely increased intracranial pressure, leading to the formation of cerebral edema have been reported in humans, especially in infants and young children after treatment with nalidixic acid (Rao, 1974). This also underlines that zebrafish larvae can show typical reactions to other influencing factors such as antibiotic treatment and can thus be used as a drug screening model not only to test the efficacy of a drug against the pathogen *in vivo* but also for the prediction of possible adverse effects on the patient.

It has been shown already, that *Cronobacter* spp. are capable to survive and replicate within human macrophages for up to 96 hours and in a recent study the FkpA protein was elucidated as a key factor involved in this ability. When using an FkpA mutant devoid in survival and replication in human macrophages, pathogenicity was strongly attenuated in the zebrafish model. This finding provides evidence that macrophages and other phagocytic cells can play a crucial role in the process of traversing physical barriers like epithelia and endothelia and by this promote further systemic spreading of intracellular bacterial pathogens within the host organism.

Taken together, by exploiting the favorable features of the zebrafish within this study, we have established and applied the first zebrafish model to study the pathogenesis of *C. turicensis in vivo.* Our model gives interesting insights into the pathogenic nature of these opportunistic facultative intracellular pathogens and shows in addition the reaction of the innate immune system to an infection in real-time. With the experimental set-up developed in this study, results can be obtained within days and the number of experiments can be scaled up easily. This means, that for example large numbers of bacterial mutants and strains can be screened for virulence related factors of *Cronobacter*, providing a promising tool to discover further detailed features of *Cronobacter* virulence and the counteracting innate immune response in future studies.

5.6 References

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Chapter 6

General Discussion and Conclusion

The central theme of my thesis was to study intracellular bacterial infections in fish with the focus of Chlamydia, both in natural fish hosts and experimentally, in the model zebrafish host. Investigating cases of epitheliocystis in populations of wild pipefish (*Sygnathus typhle*) and farmed seabream (*Sparus aurata*) in the Meditteranean further demonstrated high phylogenetic diversity of agents causing this disease. A novel member of the phylum Chlamydiae was identified in pipefish, *Ca*. Sygnamydia venezia and member of the *Simkaniaceae*, of which other family members cause respiratory infections in humans. In sea bream, the major epitheliocystis agents were two new species; *Ca*. Ichthyocystis sparus and *Ca*. Ichthyocystis helenicum, both members of a new family of proteobacteria. A novel member of the Piscichlamydia was also present, although this appeared to play a minor role in the disease. Moreover, we successfully developed two zebrafish models to study the pathogenesis of intracellular bacteria and the reaction of the innate immune system *in vivo*. By using *Waddlia chondrophila* we have established, to our knowledge, the first zebrafish model for an obligate intracellular pathogen and a member of the Chlamydiae. With the emerging human pathogen *Cronobacter turicensis* we have developed a model for infections with a facultative intracellular pathogen and a member of the proteobacteria. The demonstration that zebrafish is indeed a promising and potentially powerful model organism for studying chlamydial infections *in vivo* is particularly opportune with the rise of protocols for generating transgenic Chlamydia (Wang et al., 2011, 2013) now providing manifold opportunities to study these infectious diseases *in vivo*.

6.1 Novel epitheliocystis agents

Epitheliocystis has been described in over 90 wild and farmed fish species so far and is an emerging disease with rising importance in aquaculture, caused by a large variety of intracellular pathogens (Stride et al. 2014) Interestingly, with almost every fish species where the disease is found for the first time, a novel bacterium can be identified, indicating, that we are still only scratching the surface.

Emerging bacterial diseases like epitheliocystis are a particular problem for the aquaculture industry, where intense farming practices set fish health to greater risk of infectious diseases

(Segner et al. 2011). Aquaculture is one of the fastest growing sectors in food production with a production of 66 million tons of fish (F.A.O. 2014) per year, which corresponds to nearly 50 % of total global food fish and with an increasing need due to overfishing of wild stocks and a continuously growing world population. Gill diseases like proliverative gill inflammation (PGI) and epitheliocystis are causing serious economical losses. Because of the lack of vaccines or other preventive methods, the use of antibiotics are the treatment of choice to control these infections, which always harbours a risk for the emergence of resistant strains of pathogenic bacteria.

So far, most of the epitheliocystis agents described have been members of the phylum Chlamydiae, which is believed to having differed from other bacterial phyla already 2 billion years ago (Horn et al. 2004), in association with the occurrence of the first eukaryotes. There is evidence that the first Chlamydiae were involved in primary endosymbiosis and even into the evolution of modern plant's chloroplasts (Jensen et al. 2014), while their descendants evolved to specialised pathogens of animals, including humans. Although the most ancient known member of the phylum is the fish pathogen *Ca*. Piscichlamydia salmonis, the majority of the current knowledge of Chlamydiae derives from research into the family *Chlamydiaceae*, whose members are pathogens of land vertebrates including humans. Since fish were the only vertebrate animals for a long time in evolutionary history, it is very likely that they were also the first vertebrate hosts for chlamydial pathogens before the origin of terrestrial animals, including mammals and very recently, humans.

6.1.1 *Ca.* Syngnamydia venezia

With *Ca*. Syngnamydia venezia we have described a novel chlamydial genus and species causing epitheliocystis in Syngnathidae (pipefish and seahorses), forming with an approx. diameter of up to 400 *µ*m some of the largest single inclusions I observed during my studies. The oval-shaped inclusions that we found in the gills of the broad nosed pipefish (*Syngnathus typhle*) from the Lagoon of Venice in autumn 2011 were attached to the very tips of the epithelium of the gill lamellae. Because of their large size and the absence of pathological changes like tissue thickening or hyperplasia, we could easily dissect single inclusions under a binocular stereomicroscope free of remaining gill tissue. By PCR, cloning and sequencing of the 16S ribosomal RNA sequence we could create a phylogenetic tree which identified the causative agent as a member of the family *Simkaniaceae*, with 93-96 % nucleotide identity to other members of this family and hence, indicating a new species. We defined and named the new species *Ca*. Syngnamydia venezia, being the first described fish pathogen within this family. The morphology of the bacteria, investigated by electron microscopy, resembled partially that of other members of the Simkaniaceae with the exception, that we could not find clear evidence for the presence of EBs and hence, a biphasic life cycle of *Ca*. Syngnamydia venezia. Since with *Ca.* Piscichlamydia

salmonis another chlamydial epitheliocystis agent is probably also lacking clear EB forms, raises the question, whether the “classic” biphasic life cycle of the Chlamydiae is a later required trait and that the RBs of *Ca.* Piscichlamydia salmonis and *Ca.* Syngnamydia venezia are indeed also the infectious form of the bacteria.

The host range of the Simkaniaceae is quite broad. Other members of this family are endosymbionts of bilaterian marine worm-like creatures, insects and include with *Simkania negevensis* also a human pathogen. For this reason, the *Simkaniaceae* could be a well-suited family to study chlamydial and also host-pathogen co-evolution, starting from infections in primitive worms, which are not only lacking a brain but rather also a complex immune system, up to vertebrates, like fish and finally humans. However, this wide host range also raises the question for the zoonotic potential of these bacteria and further, whether fish other than the *Syngnathidae* can become infected. Recently, a new member of the genus, *Ca.* Syngnamydia salmonis, has been described from epitheliocystis cases of farmed Atlantic salmon in Norway (Nylund et al. 2014). The observation, that members of the *Syngnathidae* are susceptible to epitheliocystis also in a wide geographical range (Meijer et al. 2006, own documentations), raises the option of using pipefish as a possible indicator species for epitheliocystis in marine ecosystems worldwide. Due to the natural habitat of pipefish in seaweed fields, also in regions close to near-shore aquaculture production plants, it would be interesting to investigate, whether wild fish serve as a reservoir for epitheliocystis agents or if the other way round, outbreaks in aquaculture facilities could have a negative impact on wild fish populations. Furthermore, such a monitoring could also help to better estimate the risk of infectious diseases for endangered species like for example the leafy sea dragon.

6.1.2 *Ca.* Ichthyocystis spp.

In 2013 we investigated cases of epitheliocystis in farmed gilthead seabream (*Sparus aurata*). We received samples from Dr. Nancy Dourala, veterinarian and chief pathologist of the Greek aquaculture company Selonda. The gill samples originated from two outbreaks of epitheliocystis in juvenile seabream during summer and autumn of the year. The infections occurred in both cases after transfer of the fish from the land-based hatchery and early rearing facilities to the sea cages. Since the majority of the described epitheliocystis agents are members of the Chlamydiae, we started first to screen the samples for their presence and indeed most of them were PCR positive. Although cloning and sequencing of multiple 16S rRNA sequences revealed the presence of novel members of the Piscichlamydia clade and members of the genus Similichlamydia, we could not confirm the presence of these Chlamydiae inside the major lesions by FISH. After using eubacteria primers and FISH probes, we could establish that these infections were caused by two previously

unidentified beta-proteobacterial species. From 16S rRNA gene sequence, we identified a novel genus with the two distinct species, which we named *Ca.* Ichthyocystis hellenicum and *Ca*. Ichthyocystis sparus. Interestingly, the closest related organism to this new genus is *Ca*. Branchyomonas cysticola, which as well has also recently been identified as a new epitheliocystis agent in Atlantic salmon (Toenshoff et al. 2012). Although our attempts to culture the novel agents failed, we were able to perform a rather comprehensive description of the bacteria, using CLSM imaging of differently labelled inclusions, 3D FIB-SEM for morphological analysis and genomic analysis from isolated DNA of single dissected cysts. Microscopic analyses show that the intracellular inclusions harbour dividing bacteria and are surrounded by several membrane layers of surrounding epithelial cells which encapsulate the inclusions. The relatively small draft genome we obtained is comparable in size to that of many members of the obligate intracellular Chlamydiae, which indicates that these are compact, highly adapted genomes, consistent with an intracellular life style as well.

6.1.3 *In vivo* model

Although much progress has been made in the identification and description of novel epitheliocystis agents, the epidemiology of the disease remains still elusive. Some studies report a seasonal relationship or water temperature as parameters for infection (Novak & LaPatra 2006, Schmidt-Posthaus et al. 2011), but the origin and possible transmission routes of the pathogens are still unknown. The establishment of an *in vivo* model for epitheliocystis has hitherto not been feasible, because of the lack of a cultured agent of the disease. The discovery of proteobacteria as causative agents raises the possibility to eventually be able to culture a facultative intracellular member of this group. For example, a type strain of Endozoicomonas elysicola, which has been recently described as a novel epitheliocystis agent in cobia (*Rachycentrum canadum*) (Mendoza et al. 2013) grows on marine agar and is available in culture (Kurahashi and Yokota 2007).

A possible marine fish model to study epitheliocystis *in vivo* could be the *Diplodus* larva. We know from our own experiments, that we performed at the Hellenic Centre for Marine Research (HCMR) in Crete, together with Dr. Pantelis Katharios in autumn 2012, that this species (i.e. *Diplodus puntazzo*) is susceptible for epitheliocystis (see also chapter1, figure 4A). Adult fish regularly spawn in captivity and provide big amounts of eggs. Similar to the zebrafish, the Diplodus larva is small in size, widely transparent during the first 20 days of development and easy to handle under laboratory conditions.

As an already well-established model organism, the zebrafish has been applied as a tool to study infectious diseases in vertebrates and as a model for human medicine. It has recently been

proposed as a new model for aquaculture as well, which will also be discussed as a topic of the coming European Zebrafish Meeting (EZM) in Oslo this year.

6.2 Zebrafish models for infections with intracellular bacteria

Because of the lack of a cultured fish pathogen for epitheliocystis, we decided at the beginning of these studies to try to develop a zebrafish model for a chlamydial and for a proteobacterial pathogen in general. We developed a model with *Waddlia chondrophila* as a member of the Chlamydiae and an obligate intracellular pathogen and with *Cronobacter turicensis* as a member of the proteobacteria and a facultative intracellular pathogen.

6.2.1 *Waddlia* model

We chose *W. chondrophila* as a suitable candidate because of its environmental distribution in terrestrial as well as aquatic habitats and also because of its phylogenetic relationship in-between classical chlamydial pathogens of land animals including humans and the newly discovered chlamydial pathogens of fish. A further point that led us to the decision to choose *W. chondrophila,* was the susceptibility of various cell lines, including two fish epithelial cell lines, and also it's ability to replicate in phagocytic cells, such as macrophages, at least *in vitro*. The key question we wished to test *in vivo*, was which cell types were preferentially targeted by *W. chondrophila* and whether the route and manner of infection influenced this.

We have established the first zebrafish model for an obligate intracellular pathogen and a member of the Chlamydiae. We could infect zebrafish larvae via bath-immersion and microinjection technique with *W. chondrophila*, whereby we identified epithelial cells of the swim bladder, endothelial cells of the vasculature and phagocytising cells of the innate immune system as preferred targets for infection in zebrafish. *W. chondrophila* exhibited in these cells its typical appearance, consisting of a BCV containing replicating RBs and its typical assembly in association with host cell mitochondria and ER in close vicinity to the nucleus. Moreover, by using 2-5 dpf old Tg(lyzC:dsRED)^{nz50} larvae, we could exclusively observe the reaction of the innate immune system to an infection. The microinjection of *W. chondrophila* into the swim bladder evoked a strong innate immune response, characterised by the recruitment of large numbers of neutrophils to the infection site, while neutrophil recruitment is attenuated when using heat-inactivated bacteria. Intravenous injection of *W. chondrophila* into zebrafish larvae causes a sepsis with strongly impaired blood circulation and subsequent oedema formation, resulting in increased mortality. This reaction was only provoked by injection of live infectious bacteria but not by the use of heat-inactivated *W. chondrophila*. Systemic infections can be treated with tetracycline. Simple administration of the

antibiotic to the water significantly reduces the bacterial load and increases the larval survival rate. A systemic infection triggers a strong innate immune response, evidenced by the recruitment and significant depletion of phagocytes. Macrophages as well as neutrophils are recruited upon both swim bladder and intravenous injection and readily phagocytose the bacteria, whereby *W. chondrophila* demonstrates the ability to survive and even replicate inside these phagocytes. Although this shows that both innate immune cell types are susceptible to an infection by the pathogen, and can contribute to barrier crossing and further dissemination inside the host, finally, the innate immune response seems to be effective to clear the infection after 72 hpi. The recognition of *W. chondrophila* by TLRs seems to be a contributing factor to mount an innate immune response, demonstrated in MyD88 morpholino injected larvae, which showed a higher mortality and bacterial load after intravenous injection of *W. chondrophila* compared to control larvae. The contribution of MyD88 mediated signalling to neutrophil recruitment and generation of an effective early immune response has also been shown in murine models for *Chlamydia pneumoniae* infection (Rodriguez et al. 2005, Naiki et al. 2005).

The zebrafish swim bladder is an air-exposed epithelium, regarded as a homologous organ to the mammalian lung (Flores et al. 2010). Since *W. chondrophila* has been linked to cases of pneumonia in humans (Haider et al. 2008) and related organisms such as *Chlamydia pneumoniae* and *Chlamydia psittaci* can cause severe lung infections, our model could provide a new approach to investigate aspects of human respiratory disease caused by these pathogens *in vivo*. We also show for the first time that endothelial cells are susceptible for an infection with *W. chondrophila*. Infection of endothelial cells has already been shown *in vitro* for *C. pneumoniae*, which suggested that these pathogens play a possible role in the development of vascular disease like atherosclerosis (Godzik et al. 1995, Gaydos et al. 1996, Summersgill et al. 2000, Belland et al. 2004). Our findings *in vivo* for *W. chondrophila* now support a possible role for this chlamydial species and through the zebrafish model, offers an alternative for research into this question. Therefore, our model could maybe also be applied to other chlamydial pathogens, like e.g. *C. pneumonia,* to investigate and compare pathogenic features also of other species of the phylum *in vivo.* Further experiments with knockdown or knockout fish and/or larger scale transcriptomics or metabolomics approaches could additionally reveal more about chlamydial pathogenicity and Chlamydiae-host interactions *in vivo*.

6.2.2 *Cronobacter* model

A second zebrafish model was developed for an opportunistic facultative intracellular pathogen. The microinjection of as few as 50 CFU of *Cronobacter turicensis* into the yolk sac of 2 dpf old zebrafish larvae resulted in rapid multiplication of the bacteria and dissemination into the blood

stream after 24 hpi, followed by the development of a severe bacteraemia and the death of the larvae within 3 days. While the innate immune response of the embryos was sufficiently developed to control the infection after intravenous injection of up to 10^4 CFU of bacteria, it could not prevent replication and spreading of the bacteria inside the yolk, although macrophages and neutrophils were quickly recruited to the infection site, documented in realtime by live imaging. *C. turicensis* could be observed to replicate both extracellularly in the yolk and internalised in phagocytes. At 24 hpi free bacteria as well as infected macrophages were lining up near the border of the yolk sac from where a traverse into the blood circulation was observed, causing a severe bacteraemia at 30-48 hpi with bacteria colonising the lumina of small blood vessels especially accumulating in the eyes and brain. Thus, during infection in zebrafish embryos a combination of extracellular and intracellular replication of *Cronobacter* could be observed. We further investigated with our infection assay the ability of *C. turicensis* to survive and replicate in phagocytes, by testing a mutant strain lacking FkpA expression, a supposed key factor which is involved in this ability, showing strongly attenuated virulence in the larvae. The infection could very sucsessfully be treated by applicaton of nalidixic acid to the water, which significantly blocked bacterial replication and raised the survival rate of the larvae, but also caused the development of a pericardial oedema as a side effect. Other antibiotics, like ampicillin and tetracycline, which had also shown to be highly effective against *C. turicensis in vitro,* proved less effective when applied to treat infection in our zebrafish model. These findings show clearly the differences of drug efficacy *in vitro* and *in vivo* and confirm the need to test drugs for their efficacy and possible adverse effects in an *in vivo* model. Furthermore, we investigated the ability of *C. turicensis* to survive and replicate in phagocytes. They are capable of surviving and replicating within human macrophages for up to 96 hours and in a recent study the FkpA protein was shown to be a key factor involved in this ability. When using an FkpA mutant devoid in survival and replication in human macrophages, pathogenicity was strongly attenuated in the zebrafish model. These findings provide evidence that macrophages and other phagocytic cells can play a crucial role in the process of traversing physical barriers like epithelia and endothelia and by this promote further systemic dissemination of intracellular bacterial pathogens like *C. turicensis*.

A recent *in vivo* study of the opportunistic pathogen *Pseudomonas aeruginosa* in a *Caenorhabditis elegans* infection model has shown, that the pathogenicity of these bacteria rely more on basic and evolutionary conserved abilities that help the bacteria to hide from host defensive mechanisms and enable to metabolise essential nutrients inside the host rather than using obvious virulence factors like e.g. exotoxins, of specialised pathogenic bacteria (Feinbaum et al. 2012).

Therefore, future studies could exploit our model system to perform, for example, a high-throughput screening of a whole mutant library to elucidate more virulence related genes of *C. turicensis*.

6.2.3 Conclusion

For both intracellular bacteria, the survival and replication in professional phagocytes of the innate immune system like macrophages and neutrophils appears to be an important virulence factor. Moreover, these phagocytes can be utilised as a “Trojan Horse” by these bacteria to cross epithelial and endothelial barriers to invade and distribute inside the host. This is particularly crucial for the immotile *Waddlia,* while *Cronobacter* has the ability to replicate extracellularly and maybe also actively traverse cellular barriers.

A versatile new technique to answer such questions is the light sheet technology. The Z.1 light sheet microscope from Zeiss enables researchers to image whole three-dimensional living samples like a zebrafish larva in realtime with very low phototoxicity and bleaching effects. This method would be especially attractive for tracking an infection of transgenic fluorescent bacteria inside different transgenic strains of zebrafish larvae possessing fluorescent leukocyte populations or other cell types of interest. Future work to further establish the zebrafish model in infection biology could also include the development of additional mutant strains for immunity related genes and a recently applied technique to create knockout zebrafish lines is CRISPR.

Taken together, the zebrafish models for *W. chondrophila* and *C. turicensis* infection, presented in this study, are valuable tools to investigate aspects from fish to human infectious disease and immunity and supply a promising basis for future research.

6.3 References

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V CURRICULUM VITAE

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List of Publications

Alexander Fehr*, Elisabeth Walther*, Heike Schmidt-Posthaus, Lisbeth Nufer, Anthony Wilson, Miroslav Svercel, Denis Richter, Helmut Segner, Andreas Pospischil and Lloyd Vaughan; *Candidatus Syngnamydia Venezia, a Novel Member of the Phylum Chlamydiae from the Broad Nosed Pipefish, Syngnathus typhle*
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Published in PLoS one, 2013, 8(8).

Alexander Fehr, Maja Ruetten, Helena M.B. Seth-Smith, Lisbeth Nufer, Andrea Voegtlin, Angelika Lehner, Gilbert Greub, Philip S. Crosier, Stephan C. F. Neuhauss and Lloyd Vaughan; *A zebrafish larval infection model for Chlamydia: the obligate intracellular pathogen Waddlia chondrophila*
Manuscript submitted

Alexander Fehr*, Athmanya K. Eshwar*, Stephan C. F. Neuhauss, Maja Ruetten, Angelika Lehner and Lloyd Vaughan; *Evaluation of zebrafish as a model to study the pathogenesis of the opportunistic pathogen Cronobacter turicensis*
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Manuscript submitted

Helena M.B. Seth-Smith, Nancy Dourala, Alexander Fehr, Pantelis Katharios, Weihong Qi, Maja Ruetten, José M. Mateos, Lisbeth Nufer, Roseline Weilenmann, Urs Ziegler, Nicholas R Thomson, Ralph Schlapbach and Lloyd Vaughan; *Emerging epitheliocystis in farmed gilthead seabream: identification, characterisation and genomes of novel beta-proteobacterial agents*
Manuscript submitted

Holger Dill*, Bastian Linder*, Alexander Fehr and Utz Fischer; *Intronic miR-26b controls neuronal differentiation by repressing its host transcript ctdsp2*
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Published in Genes & Development, 2012, 26:25-30

Lloyd Vaughan, Alexander Fehr, Elisabeth Walther and Heike Schmidt-Posthaus; *Epitheliocystis and the importance of Chlamydia-related bacteria on fish health*
In ‘‘Free-living amoebae: an evolutionary crib for emerging pathogens’’, edited by Gilbert Greub, Blackwell Publishing (in press)

VI ACKNOWLEDGEMENTS

First of all I would like to thank Lloyd Vaughan and Stephan Neuhauss for having provided me the opportunity to work on this interesting project and their enduring support throughout my whole thesis. I've always appreciated Stephan's cheerful and open-minded nature and the manifold and very inspiring discussions with Lloyd, especially while sitting on a Mediterranean island. I also thank Lloyd for his teamwork and all of his efforts as my supervisor.

Further I would like to thank all of our collaborators and supporters of our work, especially Helmut Segner, Heike Schmidt-Posthaus and Maricruz Guevara from the FIWI in Bern, Gilbert Greub from the University Hospital in Lausanne, Pantelis Katharios from the HCMR in Crete, Angelika Lehner and Athmanya Eshwar from the Institute for Food Safety and Hygiene, Urs Ziegler, Txema Mateos and the whole ZMB team and everybody else who took part in our research.

I thank Helena Seth-Smith for her teamwork, a lot of good advice and trying to teach me proper English. I would like to thank Maja Rütten for her expert pathological descriptions and Lisbeth Nufer for her excellent performance in electron microscopy. Further I thank Roseline Weilenmann and the complete lab crew and everybody else from the Institute of Veterinary Pathology for their support.

Many thanks go to Kara Dannenhauer for taking care of the fish and providing her help in many other situations. I also thank all the other members of Stephan's lab for their help and support whenever needed.

Finally I thank all my long-standing friends and my whole family for their support and understanding during my studies.

Ich danke insbesondere meinem Vater, meiner Schwester und meiner Freundin, die mich immer unterstützt haben, vor allem in schweren Zeiten. Vielen herzlichen Dank! Grazie mille!